HEMODYNAMIC AND METABOLIC ACTIONS OF ADIPONECTIN IN MUSCLE

Carol Bussey, BSc.

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Menzies Research Institute
UNIVERSITY OF TASMANIA
HOBART

July 2011

TABLE OF CONTENTS

DECLARATION OF ORIGINALITY v
AUTHORITY OF ACCESS v
STATEMENT OF ETHICAL CONDUCT v
ABSTRACT vi
ACKNOWLEDGEMENTS viii
ABBREVIATIONS ix
PREFACE xi
Publications. xi
Oral presentations at scientific meetings. xi
Posters at scientific meetings. xi

CHAPTER 1 – INTRODUCTION 1
1.1 Overview 1
1.2 Type 2 Diabetes 1
1.3 Hemodynamic Actions of Insulin 2
1.4 Microvascular Perfusion 3
1.5 Microvascular Perfusion in Insulin Resistance 6
1.6 Adiponectin and Insulin Resistance 9
1.7 Adiponectin: Structure and Function 11
1.8 Vascular Actions of Adiponectin 15
1.9 Do acute hemodynamic actions of adiponectin contribute to insulin sensitivity? 17
CHAPTER 2 – PREPARATION AND CHARACTERISATION OF RECOMBINANT ADIPONECTIN 18
2.1 Introduction 18
2.2 Materials and Methods 20
2.2.1 Adiponectin preparation 20
2.2.1.1 Cloning the adiponectin gene 20
2.2.1.2 Protein expression 21
2.2.1.3 Purification of native protein using Ni-NTA affinity resin 22
2.2.1.4 Concentration and dialysis of protein preparations using Vivaspin 20 units 23
2.2.2 SDS-PAGE analysis 24
2.2.2.1 SDS-PAGE under denaturing conditions 24
2.2.2.2 SDS-PAGE under non-denaturing conditions 24
2.2.2.3 Staining SDS-PAGE gels for total protein 24
2.2.3 Western Blot Analysis 25
2.2.4 Preparation of mouse fat homogenate 25

2.2.5 Quantification of adiponectin by ELISA 25
2.2.6 Determination of adiponectin-mediated eNOS phosphorylation in bovine aortic endothelial cells 26
2.2.6.1 Bovine Aortic Endothelial Cell Isolation *26*
2.2.6.2 Determination of adiponectin-mediated eNOS activation *26*
2.3 Results 28
2.3.1 Analysis of the purity of the recombinant adiponectin preparation 28
2.3.2 Native conformation of recombinant adiponectin 29
2.3.3 Quantification of the recombinant adiponectin preparation 30
2.3.4 Adiponectin-stimulated eNOS phosphorylation 31
2.4 Discussion 33
CHAPTER 3 – ADIPONECTIN OPPOSES ENDOTHELIN-1-MEDIATED VASOCONSTRICTION IN THE PERFUSED RAT HINDLIMB 36
3.1 Introduction 36
3.2 Materials and Methods 39
3.2.1 Animals 39
3.2.2 Perfused rat hindlimb 39
3.2.2.1 Perfusion Buffer *39*
3.2.2.2 Surgery *39*
3.2.2.3 Perfusion apparatus *41*
3.2.2.4 Perfusion protocols *42*
3.2.2.5 Measurement of glucose and lactate *44*
3.2.2.6 Calculation of oxygen consumption *44*
3.2.3 Statistics 45
3.3 Results 46
3.3.1 Hemodynamic and metabolic actions of adiponectin 46
3.3.2 Effect of adiponectin on the hemodynamic and metabolic effects of endothelin-1 47
3.3.2.1 Adiponectin opposes vasoconstriction caused by low dose endothelin-1 ..... *47*
3.3.2.2 Adiponectin is ineffective against established endothelin-1-mediated vasoconstriction *50*
3.3.2.3 Low-dose endothelin-1 vasoconstriction is reversible *50*
3.3.2.4 Adiponectin is unable to oppose high dose endothelin-1 ..... *52*
3.3.3 Adiponectin does not effect the hemodynamic and metabolic actions of norepinephrine . 53
3.4 Discussion 54
CHAPTER 4 – MODULATION OF ADIPONECTIN AND ENDOTHELIN-1 ACTION IN INSULIN RESISTANCE 59
4.1 Introduction 59
4.2 Materials and Methods 62
4.2.1 Animals 62
4.2.2 Perfused rat hindlimb 62
4.2.3 Biochemical Measurements 64
4.2.4 Statistics 64
4.3 Results 65
4.3.1 Four week high-fat feeding causes insulin resistance 65

4.3.1.1 High-fat feeding increases adiposity *65*
4.3.1.2 High-fat feeding reduces insulin sensitivity *66*
4.3.1.3 Plasma levels of ET-1 and adiponectin following high-fat feeding *66*
4.3.2 Adiponectin is ineffective against ET-1 in insulin resistance 67
4.3.3 The response to ET-1 is reduced following high-fat feeding 69
4.3.4 Inhibition of nitric oxide synthase restores normal ET-1 response in high-fat fed rats 70
4.3.4.1 Nitric oxide synthase inhibition with L-NAME *70*
4.3.4.2 Specific inhibition of inducible nitric oxide synthase *70*
4.4 Discussion 73
CHAPTER 5 – THE HEMODYNAMIC AND METABOLIC ACTIONS OF ADIPONECTIN *IN VIVO*. 80
5.1 Introduction 80
5.2 Methods 83
5.2.1 Animals 83
5.2.2 In vivo experiments 84
5.2.2.1 Surgery *84*
5.2.2.2 Experimental procedures *84*
5.2.2.3 1-methylxanthine metabolism *86*
5.2.2.4 Muscle glucose uptake *87*
5.2.2.5 Rate of glucose turnover *88*
5.2.3 Biochemical measurements 88
5.2.4 Data analysis 89
5.2.5 Statistical analysis 89
5.3 Results 90
5.3.1 Effect of high-dose adiponectin on in vivo hemodynamics and glucose homeostasis 90
5.3.1.1 Infusion of recombinant adiponectin raised circulating adiponectin *90*
5.3.1.2 Effect of high-dose adiponectin on in vivo hemodynamics *91*
5.3.1.3 Effect of adiponectin on glucose homeostasis *93*
5.3.2 Effect of high-dose adiponectin in vivo in insulin resistance 95
5.3.2.1 High-fat feeding induces insulin resistance *96*
5.3.2.2 Effect of adiponectin on basal and insulin-mediated hemodynamics and metabolism in high-fat fed rats *96*
5.3.3 Pre-treatment and co-infusion of adiponectin during an insulin clamp revealed marked vehicle effects 100
5.3.4 Eliminating the vascular vehicle effects using a reduced dose did not expose adiponectin action 102
5.3.4.1 Adiponectin does not alter hemodynamics in vivo *102*
5.3.4.2 Adiponectin does not alter insulin sensitivity *104*
5.3.4.3 Adiponectin did not affect circulating levels of insulin or endothelin-1 ..... *105*
5.4 Discussion 107
CHAPTER 6 – DISCUSSION 112
REFERENCES 114

DECLARATION OF ORIGINALITY

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of the my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Carol Bussey

AUTHORITY OF ACCESS

This thesis may be available for loan and limited copying in accordance with the *Copyright Act 1968*.

Carol Bussey.

STATEMENT OF ETHICAL CONDUCT

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

Carol Bussey.

ABSTRACT

Insulin resistance is characterised by an impaired ability of insulin to stimulate glucose uptake, especially into skeletal muscle. Insulin has hemodynamic actions, stimulating total blood flow as well as increasing microvascular perfusion. This stimulation of microvascular perfusion may account for up to half of insulin-mediated skeletal muscle glucose uptake and is impaired in insulin resistant states. Adiponectin exhibits a number of actions similar to insulin, including stimulation of glucose uptake in incubated muscle, and reduction of glucose release from hepatocytes. Recent studies have shown that adiponectin is also able to increase nitric oxide (NO) production by the endothelium and relax pre-constricted isolated aortic rings, suggesting that adiponectin may act as a vasodilator. It was hypothesised that adiponectin may have hemodynamic effects similar to insulin, which may be an important aspect of its insulin-sensitising ability by regulating access of insulin and glucose to myocytes. Therefore, there were three major aims of this thesis: i) to examine the acute vascular actions of adiponectin in a simplified but intact vascular system, the pump-perfused rat hindlimb; ii) to investigate whether acute adiponectin infusion alters hemodynamics or glucose homeostasis in the presence and absence of insulin in anaesthetised rats; and iii) to determine whether the vascular actions of adiponectin are altered in insulin resistance.

The initial aim of the present study was to produce high purity, physiologically active recombinant adiponectin in sufficient quantity for use in perfused hindlimb and *in vivo* experiments. Full-length murine adiponectin was expressed in *E. coli* and purified by affinity chromatography. The adiponectin product was found to be present primarily as a trimer, similar to that reported in previous studies.

The constant-flow pump-perfused rat hindlimb was used to evaluate the direct effects of adiponectin on the skeletal muscle vasculature. A physiological concentration of adiponectin ($6.5 \mu g.mL^{-1}$) alone had no observable vascular activity in this largely dilated system. Adiponectin pre-treatment and co-infusion inhibited the increase in perfusion pressure and associated metabolic stimulation caused by low-dose (1nM) endothelin-1 (ET-1), but not vasoconstriction caused by either high-dose (20nM) ET-1 or 50nM norepinephrine. This action of adiponectin was apparently independent of NO, suggesting a potential novel mechanism of adiponectin action.

A high-fat fed rat model was used to determine whether the vascular actions of adiponectin are retained in insulin resistance. The ability of adiponectin to inhibit ET-1-mediated vasoconstriction was not apparent in animals fed a high-fat diet. However, the vasoconstrictor response to ET-1 (1 or 3nM) itself was found to be reduced following high-fat feeding. The vasoconstrictor response was restored in the presence of the NO synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester, indicating increased NO bioavailability during hindlimb perfusion in the insulin resistant rats. Use of a specific inhibitor of the inducible NOS (iNOS) isoform demonstrated that this increased NO was derived from induction of iNOS.

Systemic vascular and metabolic actions of adiponectin were then examined in anaesthetised rats, alone and during a hyperinsulinemic euglycemic clamp. No differences were observed between vehicle and adiponectin (24 or $96 \mu g.min^{-1}.kg^{-1}$) treated rats in macrovascular parameters, microvascular perfusion or glucose metabolism. This finding was consistent whether the rats were fed normal chow or a high-fat diet to induce insulin resistance.

This study has identified a novel vascular action of adiponectin to specifically oppose ET-1-mediated vasoconstriction. This effect was not apparent in insulin resistance, possibly

implicating loss of adiponectin activity in disease development. Additionally, the response to exogenous ET-1 was reduced in high-fat fed rats, due to upregulation of iNOS. The altered state of balance between ET-1 and NO in insulin resistance may have important implications for endothelial dysfunction. Meanwhile, no effect of acute adiponectin infusion, either hemodynamic or metabolic, was apparent *in vivo*. Whilst chronic hemodynamic actions of adiponectin may play a role in insulin sensitivity, they do not appear to be a major aspect of acute adiponectin action *in vivo*.

ACKNOWLEDGEMENTS

Sincere thanks go to my supervisors Dr. Stephen Richards and Prof. Steve Rattigan for their guidance and support. Thanks also to my associate supervisor Prof. Michael Clark for sharing his vast knowledge and enthusiasm.

I would also like to thank the rest of the Muscle Research Group, past and present, for their technical advice, helpful suggestions and moral support; including Michelle Keske, Philippe St-Pierre, Kathleen Downey, Amanda Genders, Dino Premilovac, Renee Dwyer, Cathryn Kolka, Hema Mahajan, Helena Ng, Barbara Arnts, Eloise Bradley, John Newman and Geoffery Appleby. In particular, I'd like to thank Amanda Genders for her help with the molecular biology techniques, Kathleen Downey for teaching me the perfused rat hindlimb procedure, Renee Dwyer, Philippe St-Pierre and Eloise Bradley for their extensive assistance with the *in vivo* procedures and associated analyses, Dino Premilovac for providing saline data from Sprague-Dawley rats for comparison, and Michelle Keske for providing data for fasting plasma glucose and insulin following high-fat feeding.

Special thanks to Michael Pankhurst for experimental advice, scientific discussions and sandwiches.

Thanks also to my family and friends for their love, support and patience.

ABBREVIATIONS

1-MX	1-methylxanthine
2-DG	2-deoxy glucose
ACC	Acetyl coenzyme-A carboxylase
AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
AMP	Adenosine monophosphate
AMPK	AMP-activated kinase
APPL1	Adaptor protein containing Pleckstrin homology domain, Phosphotyrosine binding domain and Leucine zipper motif 1
bp	Base pairs
BSA	Bovine serum albumin
Ca^{2+}	Calcium
$[Ca^{2+}]i$	Intracellular calcium concentration
CAMKK	Calmodulin-dependent protein kinase kinase
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin-1
FBF	Femoral blood flow
FFA	Free fatty acid
GIR	Glucose infusion rate
GLUT-4	Glucose transporter - 4
GMP	Guanidine monophosphate
HFF	High-fat fed
HGU	Hindleg glucose uptake
HMW	High molecular weight
HR	Heart rate
IMGU	Insulin-mediated glucose uptake
iNOS	Inducible nitric oxide synthase
IP_3	Inositol trisphosphate
IPTG	Isopropyl β-D-thiogalactopyranoside
LMW	Low molecular weight
L-NAME	N^G-nitro-L-arginine methyl ester

LPS	Lipopolysaccharide
kb	Kilobases
kDa	Kilodaltons
MAP	Mean arterial pressure
MMW	Mid molecular weight
NE	Norepinephrine
Ni-NTA	Ni^{2+} - nitrilotriacetic acid
NO	Nitric oxide
NF	Normal-chow fed
NOS	Nitric oxide synthase
NS	Not significant
PCR	Polymerase Chain Reaction
PI3K	Phosphatidylinositol 3-kinase
Ra	Rate of glucose appearance
Rd	Rate of glucose disappearance
R'g	Rate of muscle radioactive 2-deoxy glucose uptake
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SR	Sarcoplasmic reticulum
SREBP	Sterol regulatory element-binding protein
TNF-α	Tumour necrosis factor-α
VR	Vascular resistance
VSMC	Vascular smooth muscle cell

PREFACE

Some of the data in this thesis has been published or presented at scientific meetings and has been listed below.

Publications.

Bussey CT, Kolka CM, Rattigan S, Richards SM. (2011) Adiponectin opposes endothelin-1-mediated vasoconstriction in the perfused rat hindlimb. American Journal of Physiology Heart and Circulatory Physiology (in press).

Rattigan S, **Bussey CT**, Ross RM, Richards SM. (2007) Obesity, insulin resistance, and capillary recruitment. Microcirculation. 14(4-5):299-309.

Oral presentations at scientific meetings.

European Association for the Study of Diabetes 43rd Annual Meeting. Amsterdam, Netherlands, September 2007.

Bussey CT, Rattigan S, Clark MG, Richards SM. Insulin-like effect of adiponectin to vasodilate against endothelin-1-mediated vasoconstriction in muscle.

Sharing Excellence in Research Conference. Hobart, Australia, September 2008.

Bussey CT, Richards SM. The vascular actions of adiponectin provide a potential target for the treatment of type 2 diabetes and cardiovascular disease.

Posters at scientific meetings.

Frontiers in Vascular Medicine 2nd International Conference, Melbourne, Australia, October 2007.

Bussey CT, Rattigan S, Clark MG, Richards SM. Endothelin-1-mediated vasoconstriction in muscle is attenuated by adiponectin.

Australian Diabetes Society Annual General Meeting. Adelaide, Australia, August 2009.

Bussey CT, Kolka CM, Rattigan S, Richards SM. Adiponectin opposes endothelin-1-mediated vasoconstriction in perfused rat hindlimb by a non-nitric oxide dependent mechanism.

OzBio2010. Melbourne, Australia, September 2010.

Bussey CT, Premilovac D, Keske MA, Rattigan S, Richards SM. Low molecular weight adiponectin infusion does not alter the hemodynamic and metabolic actions of insulin in skeletal muscle in anesthetised rats.

71st Scientific Sessions of the American Diabetes Association, San Diego, CA, U.S.A., June 2011,

Bussey CT, Keske MA, Rattigan S, Richards SM. Increased inducible nitric oxide synthase activity is responsible for reduced endothelin-1 vasoconstrictor responsiveness in insulin resistance.

71st Scientific Sessions of the American Diabetes Association, San Diego, CA, U.S.A., June 2011,

Bussey CT, Premilovac D, Keske MA, Rattigan S, Richards SM. Low molecular weight adiponectin does not acutely modulate muscle hemodynamics or metabolism *in vivo*.

CHAPTER 1 – INTRODUCTION

1.1 Overview

Type 2 diabetes is a major health concern worldwide. It is caused by an inability of the body to properly respond to insulin, primarily as a decreased stimulation of glucose uptake in skeletal muscle. Insulin also stimulates both total blood flow and microvascular perfusion. Insulin-mediated microvascular perfusion has been demonstrated to be a major factor, which along with a direct action on myocytes, is involved in insulin's ability to stimulate skeletal muscle glucose uptake. This vascular action of insulin is impaired in insulin resistant states. The adipokine adiponectin has been shown to have both metabolic and vascular effects *in vitro*, and it has also been proposed to act as an insulin sensitiser. Therefore, it is hypothesised that adiponectin may have vascular activity, particularly to augment insulin-mediated microvascular perfusion, and may thus provide a novel therapeutic approach to treating insulin resistance.

1.2 Type 2 Diabetes

Diabetes mellitus is a disease of rapidly rising prevalence (Wild *et al.*, 2004). This epidemic has enormous costs, in terms of both human suffering and economic burden, and is set to be one of the major contributors to global health costs this century (Wild *et al.*, 2004; Dunstan *et al.*, 2002). Diabetes is characterised by elevated blood glucose, which results in a range of complications including neural and renal damage and cardiovascular disease (Lloyd *et al.*, 1996). More than 85% of diabetic cases are classified as type 2 (Kingwell *et al.*, 2002), caused by an inability of the body to properly respond to insulin.

Insulin is responsible for the regulation of key metabolic pathways in the liver, fat and muscle (Steinberg & Baron, 2002). Maintenance of glucose homeostasis, through the regulation of glucose release into and uptake from the blood, is a principal function of insulin. Skeletal muscle accounts for over 85% of insulin-mediated glucose uptake

(IMGU; DeFronzo *et al.*, 1985). It has been estimated that a reduced capacity for glucose uptake in skeletal muscle accounts for approximately 80% of the defect in insulin action seen in type 2 diabetics (Bonadonna, 1993). Glucose uptake in response to insulin is facilitated by the glucose transporter GLUT-4, which is translocated to the plasma membrane following stimulation (Bonadonna, 1993). This has traditionally been considered to be the primary mechanism by which insulin acts to mediate glucose uptake in skeletal muscle. However, insulin also causes dilation of blood vessels, increasing access to myocytes for both glucose and itself (reviewed in Clark *et al.*, 2003). Thus, in addition to increasing glucose transport across the cell membrane, insulin increases delivery of glucose to the cell.

1.3 Hemodynamic Actions of Insulin

Insulin is believed to cause vasodilation via a direct interaction with endothelial cells to produce nitric oxide (NO), the most potent known endogenous vasodilator (Figure 1.1; Steinberg & Baron, 2002). This occurs via a signalling cascade from the insulin receptor and insulin receptor substrate (IRS)-1, activating phosphatidylinositol 3-kinase (PI3K) and Akt, resulting in phosphorylation of endothelial nitric oxide synthase (eNOS) on serine 1177 (Kim *et al.*, 2001). Activated eNOS then catalyses the conversion of L-arginine to NO, which is released from the endothelium. The NO diffuses to nearby vascular smooth muscle cells (VSMCs), where it stimulates production of cyclic GMP, causing a reduction in intracellular Ca^{2+} and resulting in smooth muscle relaxation and vasodilation (Ignarro, 1990). Insulin-mediated stimulation of NO production has been demonstrated *in vivo* with a doubled production rate of the oxidative end-products nitrite and nitrate, accompanied by a proportional increase in leg blood flow (Baron & Clark, 1997; Steinberg *et al.*, 1994). Meanwhile, inhibition of NOS prevents insulin-mediated vasodilation, indicating that this action is NO-dependent (Steinberg *et al.*, 1994). Additionally, insulin is unable to elicit a vasodilatory response in the absence of a functional endothelium (Schroeder *et al.*, 1999). However, a study by Miller *et al.* (2002) found that insulin-mediated vasodilation in isolated mesenteric arteries was endothelium-, but not NO-, dependent, and alternately suggested a cyclooxygenase (COX) dependent mechanism. The authors suggested that variable reports may be due to differences in the size and origin

of the vessel investigated. However, that study demonstrated that insulin may elicit vasodilation by multiple mechanisms.

By stimulating vasodilation, insulin increases total blood flow (Laakso, 1990; Baron & Clark, 1997; Steinberg *et al.*, 1994), with an observed increase in cardiac output preferentially distributed to skeletal muscle (Baron & Brechtel, 1993). Stimulation of leg blood flow in humans in response to insulin closely correlates to the increase in whole body glucose uptake (Laakso *et al.*, 1992). Physically impeding rat muscle perfusion with microspheres does not affect basal glucose uptake, but markedly impairs insulin delivery to muscle and thus insulin-mediated Akt phosphorylation and glucose uptake (Vollus *et al.*, 2007). Halseth *et al.* (1998) reported that glucose delivery is a limiting factor in IMGU. Indeed, it is estimated that vascular effects account for a significant proportion of IMGU in muscle, highlighting the importance of hemodynamic actions in insulin sensitivity (Baron & Clark, 1997). Additionally, insulin-stimulated blood flow is impaired in states of insulin resistance (Laakso *et al.*, 1992; Santuré *et al.*, 2002; Wallis *et al.*, 2002). Defects in both glucose transport into myocytes and delivery to muscle have been implicated in diet-induced insulin resistance (Halseth *et al.*, 2000). Thus, increased blood flow may contribute to IMGU by enhancing delivery of both glucose and insulin to skeletal muscle, and impairments in this process may contribute to insulin resistance. However, the extent of increased total blood flow in response to insulin is insufficient to account for the large increase in muscle glucose uptake (Baron, 1994), and glucose uptake rises prior to any observable increase in total flow (Yki-Jarvinen, 1998). Furthermore, Bradley *et al.* (1999) reported that inhibition of NOS reduces glucose uptake but not total blood flow during exercise, indicating that NO augments glucose uptake independent of changes in total blood flow. Therefore, increases in total blood flow alone do not explain the NO-dependent effects of insulin to enhance muscle glucose uptake.

1.4 Microvascular Perfusion

A growing body of evidence demonstrates that insulin is able to increase microvascular perfusion within muscle, an effect termed capillary recruitment. Under basal conditions, only a proportion of all capillaries are perfused at any given time

(Sweeney & Sarelius, 1989), allowing microvascular perfusion to increase in times of increased metabolic demand, such as during exercise, hypoxia or in response to insulin (Clark *et al.*, 2003). This results in greater exposure of the tissue to glucose, and thus an increase in glucose uptake. In addition, it is thought that insulin preferentially increases flow to nutritive vessels, those that supply nutrients to muscle cells, at the expense of flow to non-nutritive vessels (Baron & Clark, 1997).

A basic premise underlying capillary recruitment is that there is a flow reserve under basal conditions, which can be redistributed when required. Much of the evidence for two functionally distinct flow routes comes from experiments using the isolated perfused rat hindlimb to study the effects of various vasoconstrictors. Some vasoconstrictors were found to enhance metabolism, including angiotensin II and vasopressin, while others, such as serotonin, inhibit it (reviewed in Clark *et al.*, 1995). Thus, although both types of vasoconstrictor increase vascular resistance, they have opposing metabolic effects. However, the changes seen in both parameters can be blocked by vasodilators, indicating that the ability of these substances to modulate metabolism is primarily due to their effects on the vasculature.

It was initially proposed that two parallel circulatory routes exist, one directly in contact with myocytes (nutritive) and another that is non-nutritive in terms of muscle, which passes through connective and adipose tissue within the muscle (reviewed in Clark *et al.*, 2000). However, fluorescently labelled microspheres infused into perfused rat hindlimbs pre-constricted with either norepinephrine or serotonin were detected throughout muscle sections with either constrictor (Vincent *et al.*, 2001b). Although norepinephrine and serotonin are thought to promote nutritive and non-nutritive flow respectively, there was no difference in the broad-scale distribution of fluorescence, indicating that the two flow routes are not anatomically separated (Vincent *et al.*, 2001b). However, the low levels of microspheres assayed within the muscle prevented description of localised variations in vessel distribution, and indeed the authors suggest that nutritive and non-nutritive vessels may exist within and outside of individual muscle fibres, respectively (Vincent *et al.*, 2001b). Additionally, physically impeding capillary blood flow impairs the metabolic actions of both type A and B constrictors (Vincent *et al.*, 2001a). In another study, microspheres small

enough to pass through resistance arterioles but not capillaries, predominantly between 8-16μm, were infused into the perfused rat hindimb. Negligible amounts of these microspheres were able to pass through the capillary bed, discounting the suggestion of large vasculature shunts bypassing the muscle (Newman *et al.*, 1996). It has alternatively been suggested that the flow routes may derive from two co-localised capillary populations; the nutritive vessels slightly narrower and longer than the non-nutritive (Harrison *et al.*, 1990). The difference in diameter between these capillaries is suggested to be as little as 2μm, but even this small difference would significantly affect the rate of blood flow and thus the opportunity for nutritive exchange (Potter & Groom, 1983). These data suggest that the two flow routes may not be anatomically separated. However, regardless of the anatomical source of the differences, it is the non-nutritive system that is thought to contain the flow reserve.

One way microvascular perfusion can be assessed is by measuring the conversion of 1-methylxanthine (1-MX) to 1-methylurate by xanthine oxidase, an enzyme concentrated in the endothelium of capillaries. To do this, exogenous 1-MX is added to the system, which was found to have no vasoactivity itself (Rattigan *et al.*, 1997a; Rattigan *et al.*, 1997b). Use of this technique in the perfused hindlimb demonstrated that there is a positive correlation between conversion of 1-MX and measures of metabolism for a wide variety of vasoconstrictors. For example, disappearance of 1-MX decreases during serotonin infusion (non-nutritive flow), coinciding with increased pressure and decreased metabolism (Rattigan *et al.*, 1997a). This was taken to indicate that the technique was measuring a change in the number of nutritive vessels exposed to the 1-MX. The same technique was then utilised *in vivo*, where physiological doses of insulin increased 1-MX metabolism by 50% with concomitant increases in blood flow and hindleg glucose uptake (HGU), indicating that insulin may act to favour nutritive flow (Rattigan *et al.*, 1997b). It has been shown that increased 1-MX metabolism is not simply a result of an increase in total flow, as infusion of epinephrine causes an increase in flow without an associated increase in 1-MX metabolism (microvascular perfusion; Rattigan *et al.*, 1997b). A physical measure of the microvasculature, by the use of contrast-enhanced ultrasound, has also been used to analyse microvascular perfusion. This method uses albumin or phospholipid microbubbles as an exclusively intravascular contrast agent to enhance the ultrasound

image and allow microvascular blood volume (MBV) to be determined. Physiological doses of insulin *in vivo* were shown to cause MBV to double using this method (Dawson *et al.*, 2002). Data obtained using these two approaches have been found to correlate well (Vincent *et al.*, 2002). Taken together, these two lines of evidence indicate that insulin mediates changes in muscle microvascular perfusion consistent with capillary recruitment.

Like glucose uptake, capillary recruitment in response to insulin precedes changes in total blood flow (Vincent *et al.*, 2002), and in fact occurs before an increase in glucose uptake can be observed (Vincent *et al.*, 2004). As the microvasculature is recruited *in vivo* within 5-10 minutes of commencement of an insulin infusion (Vincent *et al.*, 2004), this is likely to be one of the earliest actions of insulin. Capillary recruitment also occurs at lower insulin doses than those required to induce an increase total flow (Zhang *et al.*, 2004). Increased microvascular perfusion in response to insulin has also been demonstrated in the human forearm, with an associated stimulation of glucose uptake, in the absence of any change in total blood flow (Coggins *et al.*, 2001). Exercise-mediated microvascular perfusion is similarly out of proportion with the increase in total blood flow, suggesting that capillary recruitment is a distinct and separate event (Womack *et al.*, 2009). As previously stated, insulin-mediated increases in total flow have been found to correlate with whole body glucose uptake (Laakso *et al.*, 1992). However, this increased total flow does not correlate with increases in hindleg glucose uptake (HGU), a better measure of uptake into skeletal muscle (Rattigan *et al.*, 1999; Clerk *et al.*, 2002). Microvascular perfusion, on the other hand, correlates strongly with HGU, and is in fact estimated to account for up to 50% of IMGU *in vivo* (Rattigan *et al.*, 1999; Clerk *et al.*, 2002; Vincent *et al.*, 2003). Thus, via this vascular action, insulin is able to enhance perfusion of muscle independently of changes in total flow, an effect which may be extremely important in the maintenance of glucose homeostasis.

1.5 Microvascular Perfusion in Insulin Resistance

Insulin-mediated capillary recruitment is impaired in several models of insulin resistance. These include acute infusion of α-methylserotonin (Rattigan *et al.*, 1999),

tumour necrosis factor-α (TNF-α; Youd *et al.*, 2000) or lipids (Clerk *et al.*, 2002) in anaesthetised rats. These interventions have been found to block the hemodynamic actions of insulin, including 1-MX metabolism, at the same time as impairing IMGU by approximately 50%. However, neither serotonin or TNF-α blocked IMGU in incubated muscle (Rattigan *et al.*, 1993; Nolte, 1998), suggesting that, in these models, insulin resistance results largely from their actions on the vasculature. Capillary recruitment in response to insulin is absent in obese, insulin-resistant humans (Clerk *et al.*, 2006; Keske *et al.*, 2009; de Jongh, 2004), suggesting that loss of microvascular function is an early event on the path to diabetes. Furthermore, vascular insulin signalling via Akt, eNOS and NO is impaired in response to high-fat feeding prior to the development of insulin resistance in liver, muscle or adipose tissue (Kim *et al.*, 2008). Insulin-mediated capillary recruitment and associated muscle glucose uptake is also impaired in rats fed a high-fat diet (St-Pierre *et al.*, 2010), and in the Zucker obese diabetic rat (Clerk *et al.*, 2007; Wallis *et al.*, 2002). Additionally, in many cases, the defect appears to be limited to insulin signalling, as exercise-mediated capillary recruitment is retained in insulin resistance (Zhang *et al.*, 2003; Wheatley *et al.*, 2004). However, Womack *et al.* (2009) recently reported that although microvascular perfusion in response to handgrip exercise is normal in the general diabetic population it is impaired in type 2 diabetics with microvascular complications, suggesting that the exercise response may also be impaired with greater disease progression.

Insulin-mediated microvascular perfusion, along with increased total flow, is completely blocked by systemic inhibition of NOS, with an accompanying 40% impairment of IMGU (Vincent *et al.*, 2003). Local administration of a NOS inhibitor into rat hindleg also blocked insulin-mediated capillary recruitment, but not that elicited by contraction (Bradley *et al.*, 2007). These data indicates that insulin increases microvascular perfusion via a NO-dependent pathway and, in conjunction with previously described results, implicates endothelial dysfunction in the impaired vascular actions of insulin in states of insulin resistance. In addition to NO, insulin stimulates production of the vasoconstrictor endothelin-1 (ET-1; Juan *et al.*, 2004; Yang & Li, 2008), providing tightly regulated hemodynamic control under normal conditions. Imbalance in these pathways resulting in preferential vasoconstriction is

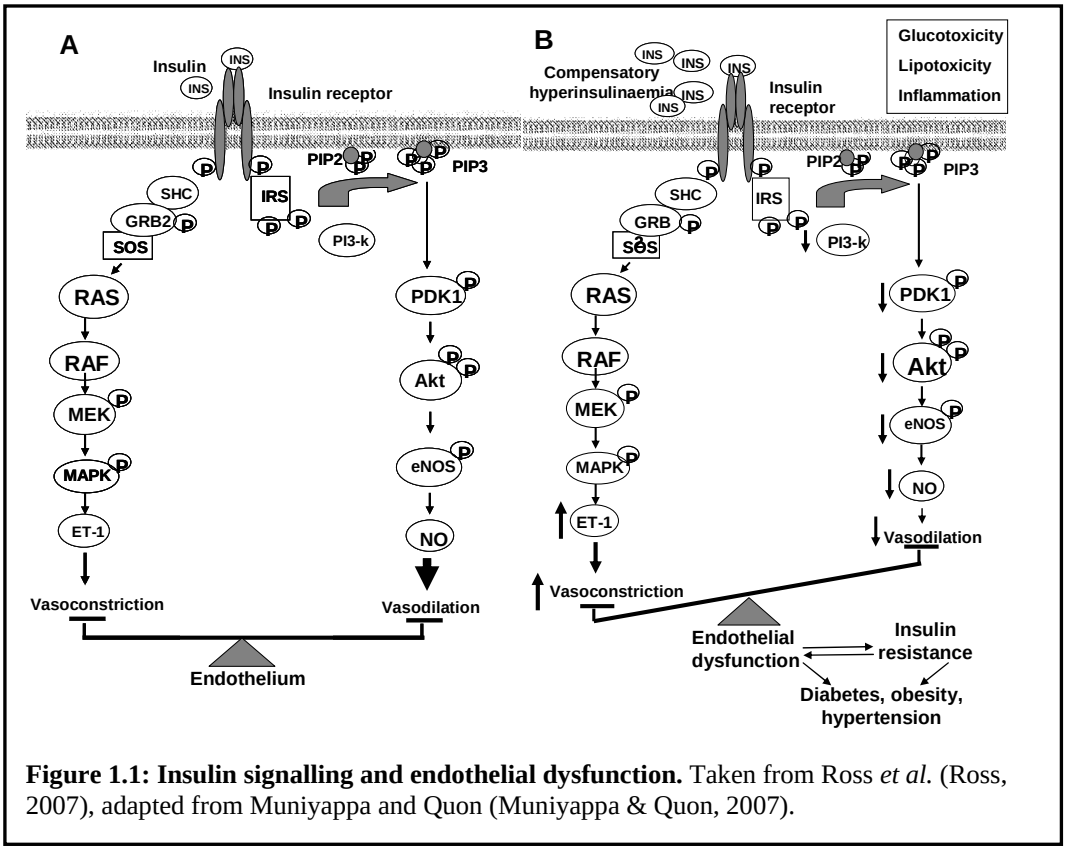

Figure 1.1: Insulin signalling and endothelial dysfunction. Taken from Ross *et al.* (Ross, 2007), adapted from Muniyappa and Quon (Muniyappa & Quon, 2007).

present in endothelial dysfunction (Figure 1.1). Romanko *et al.* (2009) described superoxide-dependent impairment of NO-mediated dilation in the fructose-fed rat model of insulin resistance in the absence of obesity, suggesting endothelial dysfunction is a primary event in the development of metabolic syndrome pathologies. Endothelial dysfunction precedes the development of type 2 diabetes (Tooke & Goh, 1998) and is believed to play an important role in the development of both microvascular (retinopathy, nephropathy, neuropathy) and macrovascular (atherosclerosis, coronary artery disease) complications of the disease (reviewed in Laight, 1999). Thus, impaired insulin-mediated capillary recruitment is potentially an important factor in the development of both diabetes and its complications.

It has been demonstrated that the hemodynamic actions of insulin are very important in maintaining glucose homeostasis, and that these effects are impaired in states of insulin resistance. Consequently, methods of augmenting insulin-mediated capillary

recruitment may provide a novel therapeutic approach to treating insulin resistance and diabetes.

1.6 Adiponectin and Insulin Resistance

Adiponectin is one of a variety of metabolically active proteins secreted by adipocytes, collectively termed adipokines (Scherer *et al.*, 1995). It is the most abundant peptide hormone circulating in plasma, with concentrations of approximately 2-20μg/mL (Arita *et al.*, 1999; Nakano *et al.*, 1996). Circulating adiponectin levels do not normally exhibit large-scale variability over a 24hr period (English *et al.*, 2003; Bertani *et al.*, 2010). However, a postprandial increase in adiponectin has been described in obese subjects, which may provide a compensatory mechanism in insulin resistance (English *et al.*, 2003). Although it is principally produced by adipose tissue, plasma levels of adiponectin decrease with increasing adiposity (Arita *et al.*, 1999; Bruce *et al.*, 2005; Kern *et al.*, 2003), unlike other adipokines such as leptin, resistin and TNF-α. Classically, it has been believed that adiponectin is exclusively expressed by adipocytes, but emerging evidence shows that it can be produced by other cells including hepatocytes and myocytes (Yoda-Murakami *et al.*, 2001; Liu *et al.*, 2009a). The functional significance of such expression has so far been unclear, but Liu *et al.* (2009a) showed that increasing skeletal muscle adiponectin expression using rosiglitazone was accompanied by significant improvements in insulin sensitivity, in both cultured myocytes and high-fat, high-sucrose fed rats.

Plasma adiponectin concentrations are reduced in subjects with diabetes and insulin resistance (Weyer *et al.*, 2001; Kern *et al.*, 2003). Similarly, serum adiponectin is decreased in mouse models of type 2 diabetes (Yamauchi *et al.*, 2001). Plasma adiponectin in humans negatively correlates with plasma glucose concentrations under fasting and hyperinsulinemic conditions, as well as basal and insulin-suppressed endogenous glucose production (Stefan *et al.*, 2003). Indeed, Yamauchi *et al.* (2001) found that adiponectin levels in mice are more closely associated with insulin sensitivity than obesity. Low plasma concentrations of adiponectin are also associated with hypertension, independent of obesity and insulin sensitivity (Iwashima *et al.*, 2004), and are found in patients with coronary artery disease (Hotta *et al.*, 2000;

Ouchi *et al.*, 1999). Adiponectin is decreased in mice fed a high-fat diet, and may be reduced within three days of high-fat feeding (Yamauchi *et al.*, 2001; Mullen *et al.*, 2009). Mutations in the adiponectin gene that reduce plasma levels are strongly associated with insulin resistance and obesity in humans (reviewed in Kadowaki & Yamauchi, 2005). Genetic deletion of adiponectin generally results in moderate insulin resistance, which is exacerbated by dietary challenge (Nawrocki *et al.*, 2006; Kubota *et al.*, 2002; Maeda *et al.*, 2002a). However, Ma *et al.* (2002) did not observe insulin resistance in adiponectin knockout mice, even after 7 months of high-fat, high-sucrose feeding. The discrepant responses may be due to compensatory mechanisms, such as increased leptin sensitivity in adiponectin knockout mice (Yano *et al.*, 2008). Adiponectin knockouts have also been shown to exhibit pro-atherosclerotic changes (Kubota *et al.*, 2002) and to develop hypertension following high-salt challenge (Ohashi *et al.*, 2006). In many cases, these changes were corrected by adenoviral replenishment of adiponectin (Maeda *et al.*, 2002a; Ohashi *et al.*, 2006).

Overexpression of adiponectin is protective against insulin resistance and atherosclerosis, and is able to offset the development of diet-induced obesity (Combs *et al.*, 2004; Satoh *et al.*, 2005; Shklyaev *et al.*, 2003; Okamoto *et al.*, 2002; Yamauchi *et al.*, 2001; Yamauchi *et al.*, 2003). Although most adipokines are pro-inflammatory and contribute to insulin resistance and endothelial dysfunction, adiponectin is anti-inflammatory and confers protection against these states (Hattori *et al.*, 2008; Yoda-Murakami *et al.*, 2001; reviewed in Lau *et al.*, 2005). Interventions that improve insulin sensitivity, including caloric restriction, weight reduction following surgical intervention and exercise training, result in increased plasma adiponectin concentrations (Berg *et al.*, 2001; Kondo *et al.*, 2009; Yang *et al.*, 2001; Kriketos, 2004; Bluher, 2006). Adiponectin is also upregulated by the insulin sensitising drugs thiazolidinediones in genetic and diet-induced models of insulin resistance, in association with ameliorated hyperglycemia, insulin resistance and hypertension (Combs *et al.*, 2002; Potenza *et al.*, 2006; Yamauchi *et al.*, 2001). Adiponectin administration causes weight loss in mice fed a high fat diet and ameliorates insulin resistance in mouse models of obesity and type 2 diabetes (Fruebis *et al.*, 2001; Yamauchi *et al.*, 2001). Collectively, these data indicate that adiponectin promotes insulin sensitivity. Therefore, a reduction in adiponectin, and thus its insulin

sensitising actions, may be an important link between the dual epidemics of obesity and diabetes.

1.7 Adiponectin: Structure and Function

Adiponectin was independently discovered and described by several groups and so was given a variety of names, including adipocyte complement-related protein of 30kDa (Acrp30; Scherer *et al.*, 1995), adipoQ (Hu *et al.*, 1996), adipose most abundant gene transcript 1 (apM1; Maeda *et al.*, 1996) and gelatine-binding protein of 28kDa (GBP28; Nakano *et al.*, 1996), although adiponectin is now the most widely accepted term.

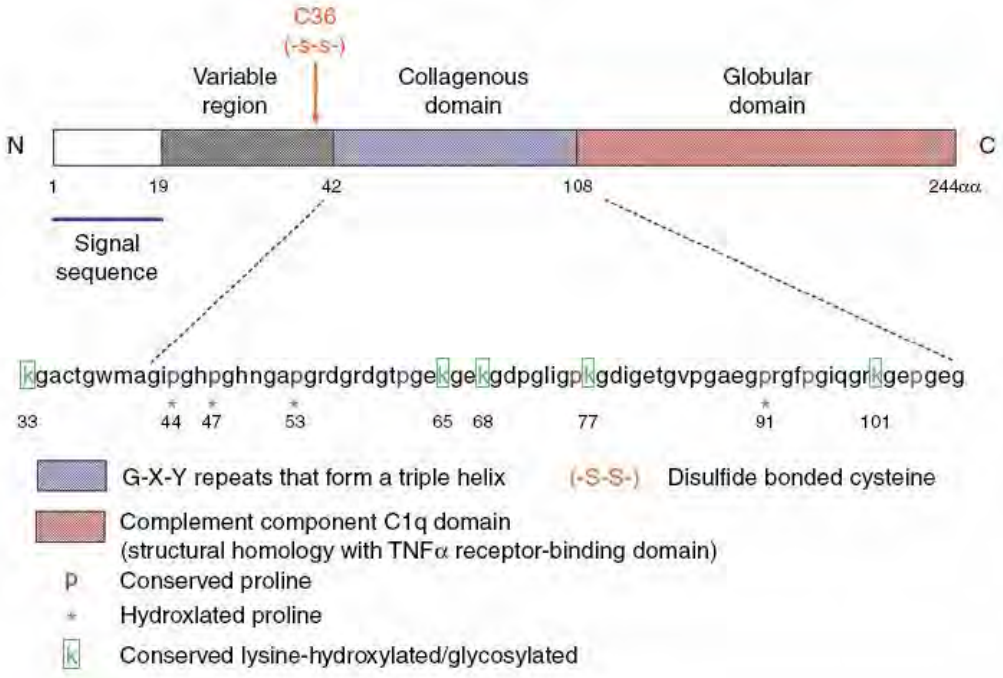

Figure 1.2: Domain structure of human adiponectin. Adapted from Whitehead *et al.* (Whitehead *et al.*, 2006).

Adiponectin is a 244 amino acid protein in humans (247 amino acids in mice) of approximately 28kDa. It consists of an amino-terminal signal peptide, a short variable region that shows no homology to other known proteins, 22 collagenous repeats involved in triple helix formation, and a globular head at the carboxyl-terminus (Figure 1.2; Scherer *et al.*, 1995; Nakano *et al.*, 1996). The protein sequence is reasonably conserved, with 91% similarity between the rat and mouse sequences, and

82% of the amino acids conserved between mice and humans (Nakano *et al.*, 1996). The majority of these conserved regions occur within the C-terminal globular domain.

The globular domain is made up predominantly of β-sheets (Shapiro & Scherer, 1998), which commonly self-associate, and so it is thought that this occurs and promotes the subsequent formation of the collagenous triple helix. Adiponectin peptides bind together via disulphide bonds and non-covalent linkages to produce a variety of homomultimers. The basic adiponectin unit *in vivo* is a trimer (low molecular weight; LMW). Hexamers (middle molecular weight; MMW) are then formed via the linking of two trimers by a disulphide bond between cysteine 36 residues (cysteine 39 in the mouse sequence; Pajvani *et al.*, 2003; Tsao *et al.*, 2003). Association of a number of hexamers leads to the formation of high molecular weight (HMW) complexes consisting of up to 18 peptides (Scherer *et al.*, 1995; Waki *et al.*, 2003). It has been demonstrated that adiponectin undergoes post-translational modification including hydroxylation and glycosylation (Wang *et al.*, 2002; Wang *et al.*, 2004), and this appears to be crucial in HMW complex formation (Wang *et al.*, 2006). This is supported by the fact that HMW species have not been observed in adiponectin expressed in bacteria, which do not carry out the post-translational modifications, and that replacement of certain amino acids involved in these modifications prevent both glycosylation and formation of HMW complexes (Waki *et al.*, 2003). All of the multimeric forms are present in plasma, although the relative proportion of each can vary (Pajvani *et al.*, 2003), and variation in the ratio of the different forms has been linked with insulin resistance (Pajvani *et al.*, 2004).

AdipoR1 and AdipoR2 are the first recognised adiponectin receptors, and may explain some of the biological actions of adiponectin (Yamauchi *et al.*, 2003). AdipoR1 is most abundantly expressed in skeletal muscle, and binds most prominently to LMW multimers of adiponectin. Meanwhile, AdipoR2 is located primarily in liver and has a higher affinity for larger adiponectin multimers. Expression of both receptors is reduced in the leptin deficient mouse model of insulin resistance (Tsuchida *et al.*, 2004). Adiponectin has been shown to upregulate the AdipoR1 receptor, an effect that was absent in obese subjects and type 2 diabetics, and did not return with weight loss (McAinch *et al.*, 2006). A third receptor, T-cadherin has been identified, which only showed affinity for hexameric and HMW adiponectin (Hug *et al.*, 2004). T-cadherin is

expressed in endothelial and smooth muscle cells, although the functional relevance of its interaction with adiponectin is not yet clear (reviewed in Takeuchi *et al.*, 2007).

A number of studies have demonstrated that adiponectin is involved in glucose homeostasis. HMW adiponectin administration lowers circulating glucose levels in mice, and Combs *et al.* (2001) found this to be entirely attributable to a reduction in hepatic glucose production Adiponectin may indirectly improve glucose homeostasis, as it has been shown to stimulate secretion of insulin (Okamoto *et al.*, 2008). However, adiponectin also has direct tissue effects. In cultured myocytes and isolated muscle, adiponectin stimulates glucose uptake, and causes a reduction in the triglyceride content of this tissue (Tomas *et al.*, 2002; Yamauchi *et al.*, 2002). Ceddia *et al.* (2005) reported that adiponectin stimulates translocation of GLUT-4 in cultured skeletal muscle cells, leading to increased glucose uptake and subsequently a reduction in glycogen synthesis. Adiponectin also stimulates phosphorylation and thus inhibition of acetyl coenzyme-A carboxylase (ACC), resulting in increased β-oxidation of fatty acids (Ceddia *et al.*, 2005; Yamauchi *et al.*, 2001; Wu *et al.*, 2003). A number of studies have shown that adiponectin operates via activation of AMP-activated protein kinase (AMPK; Ceddia *et al.*, 2005; Tomas *et al.*, 2002; Yamauchi *et al.*, 2002; Satoh *et al.*, 2005; Wu *et al.*, 2003). Chen *et al.* (2005) found adiponectin-mediated stimulation of AMPK is impaired in obese type 2 diabetics. Adiponectin stimulates AMPK phosphorylation via the adaptor protein containing pleckstrin homology domain, phosphotyrosine binding domain and leucine zipper motif (APPL1; Zhou *et al.*, 2009), a protein involved in insulin signalling and required for GLUT-4 translocation (Saito *et al.*, 2007). Recent evidence suggests a second adiponectin-activated pathway in myocytes, stimulating phospholipase C to increase intracellular calcium release, stimulate calmodulin-dependent protein kinase kinase (CAMKK) and secondarily phosphorylate AMPK (Zhou *et al.*, 2009; Hattori *et al.*, 2008). It has been demonstrated that these actions are differentially regulated by the various adiponectin multimers, with the HMW forms found to be most active in liver whilst the trimer has primarily muscular effects (Waki *et al.*, 2003).

Increasing evidence suggests that the carboxyl-terminal globular domain of adiponectin may be formed as a proteolytic cleavage product from the full-length adiponectin protein. Low levels of globular adiponectin have been identified in human

and mouse plasma (Fruebis *et al.*, 2001; Maeda *et al.*, 2002a; Yamauchi *et al.*, 2001). A potential mechanism for such a cleavage reaction, involving leukocyte elastase, has been described (Fruebis *et al.*, 2001; Waki *et al.*, 2005). This proteolysed form is unable to form multimers greater than a trimer because it lacks the cysteine residue required for disulphide bond formation (Waki *et al.*, 2003). Similar to the full-length protein, globular adiponectin has been shown to stimulate AMPK activity, fatty acid oxidation, and improve both basal and insulin-stimulated glucose uptake in isolated human skeletal muscle (Bruce *et al.*, 2005). These effects were significantly impaired in muscle from obese subjects (Bruce *et al.*, 2005). However, compared to full-length adiponectin, globular adiponectin elicits more potent activation of AMPK in muscle cells (Tomas *et al.*, 2002; Yamauchi *et al.*, 2002), greater stimulation of glucose and free fatty acid removal from plasma, and an ability to reduce the body weight of mice fed a high fat diet not seen with full-length adiponectin (Fruebis *et al.*, 2001). Additionally, the majority of studies reporting stimulation of muscle glucose uptake conducted their investigations using globular adiponectin (Bruce *et al.*, 2005; Ceddia *et al.*, 2005; Tomas *et al.*, 2002; Wu *et al.*, 2003). Therefore, it has been proposed that globular adiponectin may be the most potent adiponectin species in muscle.

Overall, the metabolic actions of adiponectin are somewhat complex, but can be summarised as shown in Figure 1.3.

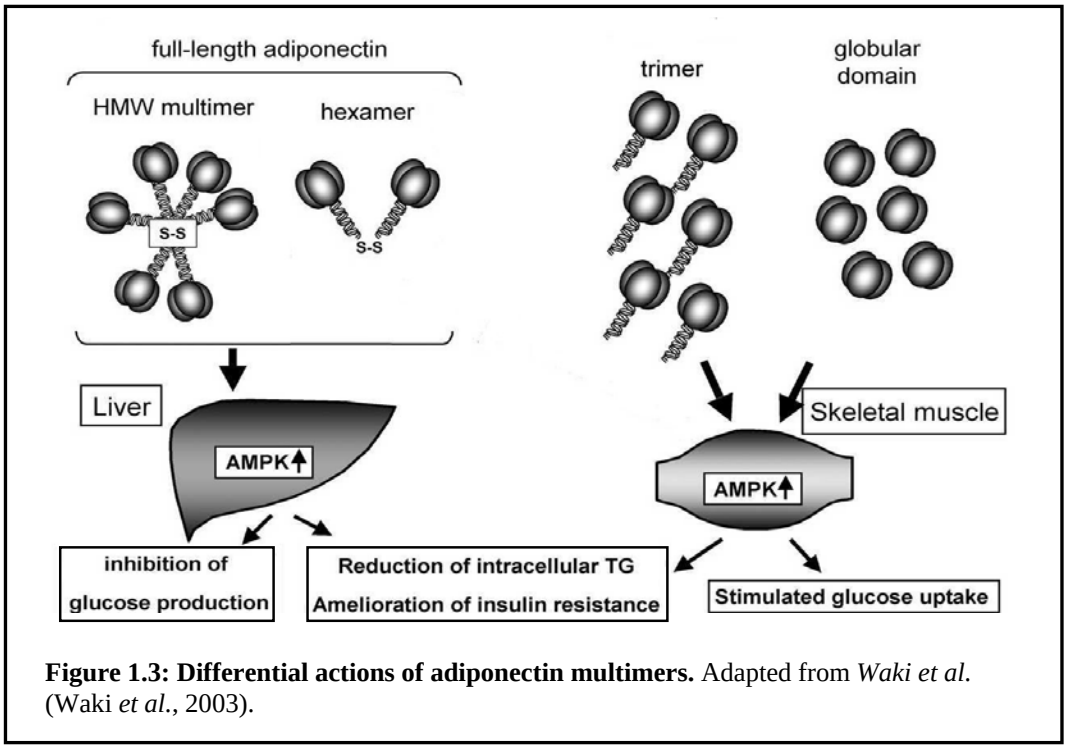

Figure 1.3: Differential actions of adiponectin multimers. Adapted from *Waki et al.* (Waki *et al.*, 2003).

1.8 Vascular Actions of Adiponectin

A growing body of evidence indicates that in addition to its direct insulin-sensitising effects, adiponectin is active in the vasculature. Decreased levels of adiponectin are associated with hypertension (Iwashima *et al.*, 2004) and coronary artery disease (Ouchi *et al.*, 1999), as well as atherosclerosis and vascular dysfunction (reviewed in Goldstein & Scalia, 2004). Hypoadiponectinemia is strongly associated with impaired vasoreactivity in hypertensive patients (Ouchi *et al.*, 2003). As stated above, adiponectin confers protection against inflammation and endothelial dysfunction (reviewed in Lau *et al.*, 2005). Adiponectin is cardioprotective, reducing cell death and oxidative stress associated with myocardial ischemia/reperfusion injury (Tao *et al.*, 2007). These effects have been demonstrated to occur via stimulation of AMPK, Akt and NO production (Gonon *et al.*, 2008). Adiponectin suppresses smooth muscle cell proliferation (Wang *et al.*, 2005) and inhibits monocyte adhesion to cultured endothelial cells (Ouchi *et al.*, 1999; Ouchi *et al.*, 2001), revealing anti-atherosclerotic properties. Indeed, Yamauchi *et al.* (2003) found that overexpression of globular adiponectin protects against the formation of atherosclerotic lesions in the apolipoprotein E-deficient mouse. Adiponectin also stimulates angiogenesis, via a mechanism involving AMPK, PI3K and Akt (Ouchi *et al.*, 2004). Increases in adiponectin due to caloric restriction are associated with improved revascularisation of ischemic rat hindlimb, via an AMPK and eNOS dependent pathway (Kondo *et al.*, 2009). Another study demonstrated adiponectin-stimulated revascularisation in ischemic muscle via a PI3K, Akt and COX2 dependent mechanism (Ohashi *et al.*, 2009). This action was found to be occurring via adiponectin interacting with calreticulin and its adaptor protein CD91, rather than the previously described adiponectin receptors (Ohashi *et al.*, 2009). This novel receptor pathway involves non-classical ligand binding, so may require the high adiponectin concentrations that occur *in vivo*. The authors found that the effects of adiponectin on eNOS and COX2 are additive, and vascular endothelial cell function depends on the products of both reactions (Ohashi *et al.*, 2009).

A role for adiponectin in hemodynamic regulation has previously been suggested by a correlation between increased adiponectin in liver cirrhosis and increased blood flow

and vascular resistance (Tietge *et al.*, 2004). Plasma adiponectin in type 2 diabetics also correlates with insulin-stimulated NOS activity and whole body glucose uptake (Kashyap *et al.*, 2010). Low levels of plasma adiponectin in humans are associated with impaired endothelium-dependent vasodilation, independent of type 2 diabetes (Tan *et al.*, 2004). Similarly, endothelium-dependent vasodilation is impaired in adiponectin knock-out mice (Ouchi *et al.*, 2003). Several groups have demonstrated increased production of the vasodilator NO by endothelial cells in response to both globular and full-length mammalian adiponectin (Chen *et al.*, 2003; Hattori *et al.*, 2003; Xi *et al.*, 2005). This adiponectin-stimulated NO production is mediated by activation of PI3K and AMPK, leading to induction of eNOS phosphorylation and eNOS- heat shock protein 90 (HSP90)-Akt complex formation (Chen *et al.*, 2003; Xi *et al.*, 2005). Interestingly, chemical activation of AMPK has previously been found to cause microvascular recruitment (Bradley *et al.*, 2010). Indeed, Cheng *et al.* (2007) described APPL1 and AMPK dependent adiponectin-meditated vasorelaxation in mesenteric arteries from lean heterozygous leptin deficient mice. This effect was endothelium-dependent and abolished by NOS inhibition, emphasising the likely importance of NO in adiponectin-induced vasodilation (Cheng *et al.*, 2007). Additionally, adiponectin-meditated vasorelaxation was significantly reduced in arteries from homozygous leptin deficient diabetic mice, and the authors suggest that reduced function of adiponectin and/or APPL1 may contribute to endothelial dysfunction (Cheng *et al.*, 2007). Xi *et al.*, 2005) have also demonstrated NO- and endothelium-dependent relaxation of pre-constricted isolated aortic rings in response to globular adiponectin. Furthermore, Fesus *et al.* (2007) found that human recombinant adiponectin blunted serotonin-mediated vasoconstriction in isolated aortic rings and mesenteric arteries, an effect which was endothelium-independent in contrast to previous studies. These observations suggest that in addition to direct affects on skeletal myocyte glucose uptake, adiponectin may enhance glucose delivery by stimulating vasodilation.

As described above, NO-dependent vasodilator activity of insulin is a major aspect of its ability to stimulate glucose uptake. Thus, any similar responses adiponectin elicits may make important contributions to both its insulin sensitivity and cardiovascular benefits.

1.9 Do acute hemodynamic actions of adiponectin contribute to insulin sensitivity?

Hemodynamic regulation is a major aspect of insulin action. These effects are impaired in insulin resistance, and alternative methods of stimulating it may be therapeutically beneficial in insulin resistance and type 2 diabetes. Adiponectin shows potential to have similar vascular actions to insulin, which may improve glucose homeostasis by regulating access of insulin and glucose to myocytes. However, the effects of adiponectin have not been examined in an intact vascular system, and so the physiological significance of such effects remains unclear. Therefore the overall aim of this thesis was to determine whether adiponectin acts in the vasculature acutely to improve insulin sensitivity.

This principal aim was addressed via four major research aims:

i) to produce physiologically active recombinant adiponectin;

ii) to examine the acute vascular actions of adiponectin in a simplified but intact vascular system, the pump-perfused rat hindlimb;

iii) to investigate whether acute adiponectin infusion alters hemodynamics and/or glucose homeostasis in the presence and absence of insulin in anaesthetised rats;

iv) to determine whether the acute vascular actions of adiponectin are altered in insulin resistance.

CHAPTER 2 – PREPARATION AND CHARACTERISATION OF RECOMBINANT ADIPONECTIN

2.1 Introduction

In order to undertake investigations into the physiological actions of adiponectin, a supply of adiponectin was first required. While recombinant adiponectin is commercially available, the cost of the large amounts required for perfused rat hindlimb and *in vivo* experiments is prohibitive, with micrograms required to maintain physiologically relevant ($\mu g.mL^{-1}$) doses throughout the experiments (Fesus *et al.*, 2007; Cheng *et al.*, 2007). Recombinant protein expression in *E. coli* is a widely used method of producing large quantities of a desired protein (Kou *et al.*, 2007; Galluccio *et al.*, 2007). Although mammalian and bacterially produced proteins can vary in their actions, much lower protein yields are achieved with mammalian cells. Thus bacterial expression was considered the most appropriate system to produce the required amounts of adiponectin.

Adiponectin circulates as low molecular weight (LMW), mid molecular weight (MMW) and high molecular weight (HMW) multimers (Shklyaev *et al.*, 2003). A number of studies have identified differences in the biological actions of the various adiponectin multimeric forms (reviewed in Whitehead *et al.*, 2006). LMW adiponectin acts predominantly via AdipoR1 receptors in skeletal muscle while larger adiponectin multimers have a higher affinity for the AdipoR2 receptors located primarily in liver (for review see Kadowaki & Yamauchi, 2005). Therefore, the large multimeric complexes formed by mammalian-produced adiponectin have well described hepatic actions but low potency in muscle (Tsao *et al.*, 2003; Waki *et al.*, 2003). Conversely, LMW and globular adiponectin have been found to have greater action on muscle (Yamauchi *et al.*, 2001; Yamauchi *et al.*, 2002; Fruebis *et al.*, 2001; Tomas *et al.*, 2002; Waki *et al.*, 2003). The present study focused on the effects of adiponectin in skeletal muscle, and the bulk of evidence suggests this is primarily a target of LMW

adiponectin. Therefore, bacterially expressed LMW and globular adiponectin were selected for use in the current investigations.

The aim of this group of experiments was to produce purified recombinant adiponectin, with four major requirements, i) accuracy of sequence and structure, ii) adequate yield, iii) sufficient purity, and iv) biological activity. This required cloning the adiponectin gene sequence, protein expression in *E. coli,* purification of the recombinant protein and quantification of the protein product. The protein product was then quantified and characterised via SDS-PAGE and Western Blot for purity and multimerisation status. The recombinant adiponectin was shown to stimulate phosphorylation of eNOS in isolated endothelial cells, indicating that it is biologically active.

2.2 Materials and Methods

2.2.1Adiponectin preparation

2.2.1.1 Cloning the adiponectin gene

The full-length and globular adiponectin sequences were cloned separately for bacterial expression. Total RNA was extracted from differentiated 3T3-L1 adipocytes using the TRI Reagent® method (Sigma, St Louis, MO, USA). Approximately 1ng of total RNA was reverse transcribed using SuperScript™ III RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The mouse full-length adiponectin cDNA excluding the N-terminal signal sequence (718bp; Gen bank accession number NM_009605) was amplified, using the 5'-CACCAGTCATGCCGAAGATGA-3' sense and 5'-ATGGGTAGTTGCAGTCAGTTGG-3' antisense primers. Platinum® Pfx polymerase (Invitrogen, Carlsbad, CA, USA) was used to produce a blunt-ended PCR product. This sequence was incorporated into the pET100/D-TOPO bacterial expression vector (Figure 2.1; Invitrogen, Carlsbad, CA, USA).This vector includes a polyhistidine tag, allowing simple purification of the protein using a metal chelating resin. This tag is situated on the N-terminus, which is imperative since the C-terminal end of adiponectin is highly conserved and thought to mediate many of the protein's important actions (Fruebis *et al.*, 2001), so any modification to this region was likely to affect the function of the protein. The sequence for the globular domain of adiponectin, corresponding to amino acids 110-248 of murine adiponectin (483bp) was amplified from the previously prepared adiponectin plasmid using 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGCCGCTTATATGTATCGCT-3' sense and 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTGCAGTCAGTTGGTATCATGG-3' antisense primers, including the attB sequence for insertion into the pEXP1-DEST expression vector (Invitrogen, Carlsbad, CA, USA).This vector has similar features and an identical N-terminal tag to the pET100/D-TOPO vector.

The pET/D/Acrp plasmid was transformed into OneShot® TOP10 Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA, USA) for proliferation and purified using the Wizard®*Plus* SV Minipreps DNA Purification System (Promega, Madison, WI, USA). Plasmid fidelity and orientation was confirmed by i) size analysis via

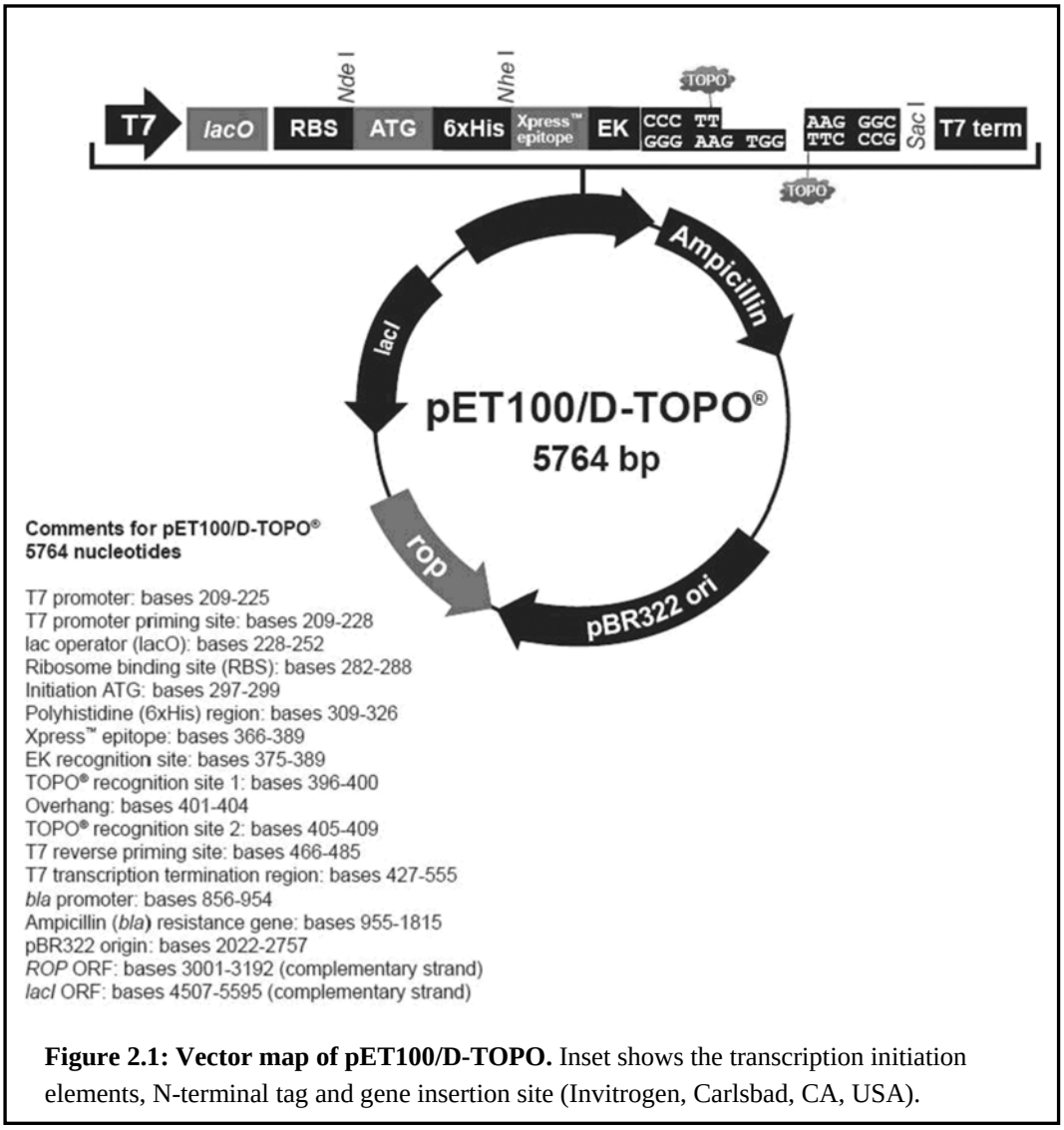

Figure 2.1: Vector map of pET100/D-TOPO. Inset shows the transcription initiation elements, N-terminal tag and gene insertion site (Invitrogen, Carlsbad, CA, USA).

electrophoresis following linearisation with the HindIII restriction enzyme, ii) PCR amplification of the regions overlapping each insertion site, and iii) sequencing using the CEQ™ DTCS-Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) according to the manufacturer's instructions, except that the amount of glycogen added with the stop solution was halved, as it had previously been determined in this laboratory that lower levels improve the efficiency of sequencing.

2.2.1.2 Protein expression

Expression of the adiponectin gene from the pET/D/Acrp plasmid occurs via a T7 promoter, under the control of the *lac* operon, which permits efficient expression of the target gene in *E. coli*. The *lac* repressor, coded for by the *lac* ORF, binds to the *lac* operon controlling the T7 promoter and inhibits basal expression of the recombinant

protein. When isopropyl β-D-thiogalactopyranoside (IPTG) is added to the bacterial culture it competes with the lac repressor, removing it from the lacO site and allowing transcription to proceed. The BL21(DE3) *E. coli* strain carries the DE3 bacteriophage lambda (λDE3) lysogen and is specifically designed for expression of genes regulated by the T7 promoter. The λDE3 lysogen contains the gene for T7 RNA polymerase, under the control of the lacUV5 promoter, which operates as described above. The promoters for T7 RNA polymerase do not naturally occur in *E. coli* and the enzyme is highly specific (Studier & Moffatt, 1986), so the polymerase will only transcribe introduced T7-regulated genes. Proteases that break down heterologous proteins are absent from B strains of E. coli such as BL21(DE3) (Grodberg & Dunn, 1988), ensuring that neither the T7 RNA polymerase nor the target protein are proteolysed. Thus, when IPTG is added to cultures of this bacterium T7 RNA polymerase is produced and repression of the target gene is removed, providing a dual control mechanism to ensure strict control of protein expression.

BL21(DE3) *E. coli* (Invitrogen, Carlsbad, CA, USA) were made chemically competent using $CaCl_2$ in order to facilitate uptake of DNA. The plasmid sample was added to competent cells and gently stirred using a pipette tip to limit cell lysis. Transformed bacteria were aliquoted and stored at -80°C in 15% glycerol. Transformed bacteria were grown up to log phase (0.5-0.8 OD) before IPTG was added to a final concentration of 6.7mM to induce protein expression. The amount of IPTG was increased from the 1mM suggested by the manufacturer following variable results in pilot expression experiments. Bacteria were grown for a further 3hs, which produced optimal adiponectin expression, before the cells were harvested. A vehicle preparation was produced using untransformed BL21(DE3) *E. coli* and without the addition of IPTG, whilst all other processes remained the same.

2.2.1.3 Purification of native protein using Ni-NTA affinity resin

Metal chelate affinity chromatography is a common method of protein purification, using a substrate such as Ni-NTA resin, composed of Ni^{2+} ions bound to nitrilotriacetic acid (for review see Gaberc-Porekar & Menart, 2005). The imidazole rings of closely associated histidine residues bind to the nickel ions and consequently proteins that contain them are immobilised in the resin. Often, as in this study, a

polyhistidine tag is introduced to a target protein by recombination with a vector that contains the corresponding nucleotide sequence. As it is uncommon to find stretches of several neighbouring histidine residues in naturally occurring proteins, this technique is reasonably selective.

Recombinant adiponectin was purified under native conditions using the Ni-NTA Purification System from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the bacteria were lysed by a combination of lysozyme and a series of rapid sonicate-freeze-thaw cycles. The bacterial lysate was bound to the Ni-NTA resin for 1hr with gentle agitation. Unbound protein was removed by a series of washes in low ionic strength imidazole buffer. The recombinant protein was eluted in 15mL of 250mM imidazole and stored at 4°C.

2.2.1.4 Concentration and dialysis of protein preparations using Vivaspin 20 units

After purification using a Ni-NTA column, the recombinant protein is present in a high concentration imidazole buffer. Imidazole and chemicals containing an imidazole ring have been found to have numerous effects *in vivo*; including inhibition of mitogen-activated protein kinases, resulting in the reduced production of pro-inflammatory cytokines such as interleukin 1 and TNF-α (English & Cobb, 2002), and activation of some phosphodiesterases (Gaillard, 1970). As these molecules exhibit vasoactivity, the presence of imidazole could affect the outcomes of the current studies. Thus it was necessary to remove the imidazole from the recombinant adiponectin.

Samples were concentrated and dialysed with phosphate buffered saline (0.14M NaCl, 2.5mM KCl, 1.5mM KH2PO4, 10mM Na2HPO4; pH 7.4) using Vivaspin 20 ultrafiltration units (Sartorius, Goettingen, Germany). Total protein content was assessed using the Lowry method (Lowry *et al.*, 1951; DC Protein Assay, Bio-Rad, Hercules, CA, USA). Adiponectin preparations were stored at 4°C for up to 30 days.

2.2.2 SDS-PAGE analysis

2.2.2.1 SDS-PAGE under denaturing conditions

Protein samples were analysed via denaturing SDS-PAGE using the XCell *SureLock*™ Mini-Cell and NuPAGE® Novex Pre-Cast 1.0 mm, 10 well, 10% Bis-Tris Gels (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. NuPAGE® Antioxidant was used to prevent proteins reoxidising during electrophoresis. Samples were combined with an equal volume of 2x SDS-PAGE Sample Buffer (0.125M Tris-HCl pH 6.8, 20% glycerol, 4% β-mercaptoethanol, 0.2%Bromophenol Blue, 4% SDS). Samples were heated at 90°C for 5-10 minutes to denature the protein, immediately before being loaded onto a SDS-PAGE gel. Each gel was loaded with 5μL of the SeeBlue® Plus2 Pre-Stained Standard (Invitrogen, Carlsbad, CA, USA) and 20μL of each sample to be analysed. The gels were electrophoresed at 200 volts for 45-60 minutes at room temperature.

2.2.2.2 SDS-PAGE under non-denaturing conditions

In order to examine the native conformation of the recombinant adiponectin, samples were analysed via non-denaturing SDS-PAGE using 3-8% Tris-Acetate NuPAGE® gels (Invitrogen, Carlsbad, CA, USA). Samples were prepared in buffer containing no β-mercaptoethanol (LDS Sample Buffer; Invitrogen, Carlsbad, CA, USA) and were not heated, as these treatments break disulphide bonds between cysteine residues and non-covalent bonds between protein subunits, respectively. Prepared samples (20μL) were loaded onto a gel, along with 5μL of the SeeBlue® Plus2 Pre-Stained Standard. The buffer chambers were filled with Tris-glycine Native Running Buffer (Invitrogen, Carlsbad, CA, USA) with no added antioxidant, and the gel was electrophoresed at 150 volts for 1-2hrs.

2.2.2.3 Staining SDS-PAGE gels for total protein

SDS-PAGE gels were soaked in Gel Staining Solution (0.1% coomassie blue, 25% methanol, 10% acetic acid) overnight to stain for protein. The gels were then soaked in Gel Destaining Solution (20% methanol, 10% acetic acid) for 4-8hrs until satisfactory destaining was achieved.

2.2.3 Western Blot Analysis

Following separation via non-denaturing SDS-PAGE, proteins were transferred to nitrocellulose membranes at 30 volts for 1hr at room temperature. The membranes were washed in TBS (0.02MTris base, 0.137MNaCl; pH 7.6), then incubated in Blocking Buffer (5% non-fat milk powder in TBS/T) for 1hr at room temperature.

Immunoblotting was conducted with either a specific anti-adiponectin antibody (AB3269P; Chemicon, Temecula, CA, USA) or the Anti-Xpress™ antibody, which recognises an epitope in the N-terminal tag (Invitrogen, Carlsbad, CA, USA). The membrane was placed into a solution of either antibody at a dilution of 1:5000 in Blocking Buffer and tumbled at 4°C overnight. The membrane was washed three times in TBS/T (0.1% Tween 20 in TBS); before being tumbled for 1hr at room temperature with an appropriate secondary antibody linked to horseradish peroxidise (Cell Signalling, Danvers, MA, USA). The membrane was again washed in TBS/T, before immunoreactive proteins were visualised using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA). Chemiluminescence was detected by exposure to the blot and subsequent development of autoradiography film (Amersham (GE Healthcare)).

2.2.4 Preparation of mouse fat homogenate

Mouse fat homogenate was obtained as source of mammalian adiponectin for comparison with the bacterially expressed recombinant adiponectin produced in this study. The epididymal fat pads from five male mice were pooled and ground to a powder under liquid nitrogen. Six millilitres of Sample Buffer (0.02M Tris base pH 7.5, 0.2M potassium fluoride, 0.02M EDTA) containing 50µL of Protease Inhibitor Cocktail (Sigma, St Louis, MO, USA) was added to approximately 1g of tissue in a chilled tube. The sample was homogenised using an ultraturrax, before being centrifuged at 13,000g for 10min at 4°C in a microcentrifuge. The infranatant was carefully collected, as adipocytes produce a top layer of fat from this process. This sample was aliquoted and frozen in liquid nitrogen, before being stored at -80°C.

2.2.5 Quantification of adiponectin by ELISA

Adiponectin concentrations were assayed using the Adiponectin ELISA kit from Phoenix Pharmaceuticals (Burlingame, CA, USA), according to the manufacturer's

instructions. This assay was specifically chosen as it reacts with both mouse and rat adiponectin, and so would detect both the recombinant mouse adiponectin and adiponectin endogenous to the rats studied. Samples were serially diluted until they were within the range of the standard curve.

2.2.6 Determination of adiponectin-mediated eNOS phosphorylation in bovine aortic endothelial cells

2.2.6.1 Bovine Aortic Endothelial Cell Isolation

Bovine aortic endothelial cells (BAECs) were isolated from thoracic aortae excised from one year old calves. Aortae were placed in sterile HEPES Buffered Hanks Balanced Salt Solution (HBSS; in mM: 136.9 NaCl, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 5.37 KCl, 4.16 $NaHCO_3$, 5.55 *D*-glucose, 0.027 Phenol Red, 20.0 HEPES, 1.26 $CaCl_2$, 0.81 $MgSO_4$) and transported to the laboratory within 1hr of excision. The fatty adventia was removed from each section. The lumen was exposed and rinsed thoroughly with HBSS to remove adherent blood or gut bacteria. The endothelial layer was gently collected into HBSS containing 10μg.mL^{-1} gentamicin and 2.5μg.mL^{-1} amphotericin (both Sigma, St Louis, MO, USA) and kept on ice. The cells were washed in HBSS, and suspended in 0.1% Collagenase II (Worthington, Lakewood, NJ, USA) in HBSS. The suspension was incubated at 37°C for 15min with trituration, before digestion was neutralised by the addition of Foetal Calf Serum (FCS; JRH Biosciences, Lenexa, KS, USA). The cells were collected by centrifugation at 300g and resuspended in sterile low glucose Dulbecco's Modified Eagles Medium (DMEM; pH 7.4; GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 44mM $NaHCO_3$, 10mL.L^{-1} penicillin/streptomycin solution (JRH Biosciences, Lenexa, KS, USA), 4mM *L*-glutamine (Sigma, St Louis, MO, USA) and 10% FCS. BAECs were incubated in a humidified, 5% CO_2 atmosphere at 37°C and passaged every 2-4 days. Cells were not passaged more than five times, as after this the morphology and thus possibly the phenotype of the cells is altered by the culturing process.

2.2.6.2 Determination of adiponectin-mediated eNOS activation

Cells were serum starved for 2hr in Krebs Buffer (120mM NaCl, 4.7mM KCl, 1.2mM KH_2PO_4, 2.4mM $MgSO_4$, 25mM $NaHCO_3$, 1.25mM $CaCl_2$). The cells were then incubated for 15min with adiponectin (0-10μg.mL^{-1}) diluted in Krebs. The buffer was

aspirated and the cells resuspended in Solubilising Buffer (0.12mM Tris-HCl, 4% w/v SDS, 20% glycerol, 10% β-mercaptoethanol, Bromophenol Blue, pH 6.8) with thorough trituration. Samples were subjected to non-denaturing SDS-PAGE and Western Blotting as described in Sections 2.2.2 and 2.2.3, except that electrophoretic transfer to nitrocellulose was conducted at 30 volts at 4°C overnight. Membranes were immunoblotted using antibodies for total or serine 1177 phosphorylated eNOS (BD Transduction Laboratories, Franklin Lakes, NJ, USA) diluted in 5% BSA in TBS/T.

2.3 Results

2.3.1 Analysis of the purity of the recombinant adiponectin preparation

To verify the purity of the adiponectin, a SDS-PAGE was conducted on fractions eluted from a Ni-NTA column and stained for total protein. A distinct band was apparent in each of the fractions at 29.4kDa, the expected size of adiponectin, whilst the remainder of the gel was essentially free from protein (Figure 2.2). Coomassie blue stained SDS-PAGE of globular adiponectin similarly showed enrichment of a band at 20.7kDa, the predicted size of the recombinant protein, following purification (data not shown). This indicated that recombinant adiponectin had been purified from the bacterial lysate. Yield was highest in the initial fraction and rapidly reduced with increasing elution volume. All experimental adiponectin preparations were prepared from 12-15mL elution volumes.

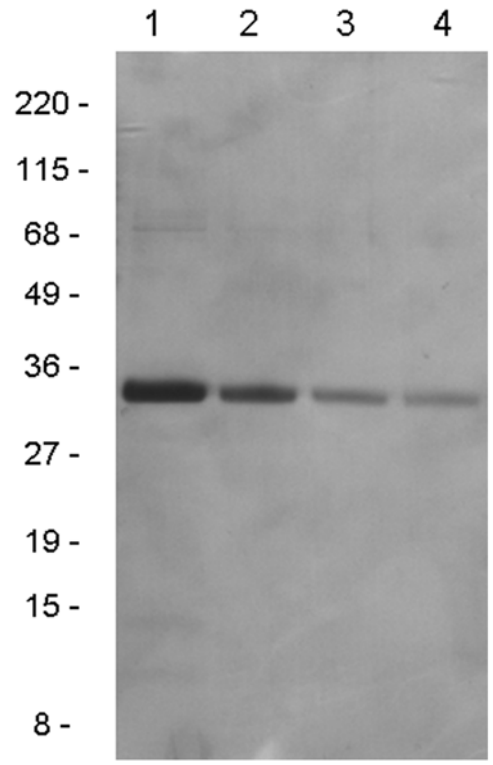

Figure 2.2: De-naturing SDS-PAGE of purified adiponectin fractions stained for total protein. Lanes 1-4 show samples of consecutive 1mL fractions eluted from a Ni-NTA column. Predicted size of the monomeric recombinant protein was 29.4kDa.

Both the full-length and globular proteins were confirmed as recombinant adiponectin through immunoreactivity with both the anti-Xpress antibody, which detects the N-terminal tag, and with the specific anti-adiponectin antibody. Meanwhile, no immunoreactive bands were detected in vehicle preparations (Figure 2.3B), indicating that they were free from recombinant protein as expected.

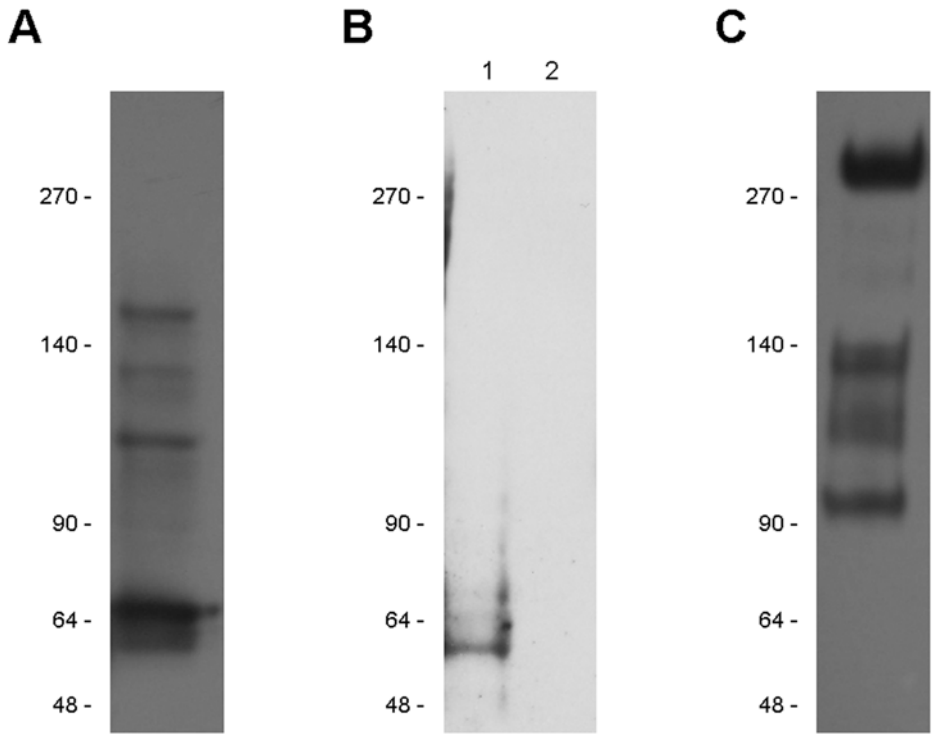

Figure 2.3: Native conformation of recombinant adiponectin preparations. Non-reducing, non-denaturing Western Blot analysis of (A) full-length recombinant adiponectin, (B) recombinant globular adiponectin (Lane 1) and vehicle (Lane 2), and (C) mammalian adiponectin. A and C were conducted using the anti-adiponectin antibody, whilst B was prepared using the anti-Xpress antibody.

2.3.2 Native conformation of recombinant adiponectin

The distribution of the multimeric forms of recombinant adiponectin was evaluated via non-denaturing, non-reducing SDS-PAGE and Western blotting (Figure 2.3). The bacterially produced full-length adiponectin was present substantially at an apparent weight of approximately 70kDa, the reported weight of the trimer (Figure 2.3A). Small amounts of immunoreactive protein was also observed between approximately115-150kDa, consistent with mid molecular weight adiponectin species, which have previously been reported in bacterially expressed adiponectin (Figure

2.3A; Waki *et al.*, 2003; Yamauchi *et al.*, 2002; Tsao *et al.*, 2002). The apparent weight of trimeric adiponectin has previously been reported to be lower than its true molecular weight, as the quarternary structure of the non-denatured protein affects the way it travels through the gel (Waki *et al.*, 2003). Native recombinant globular adiponectin appeared as a single band at approximately 60kDa, consistent with a trimer, but lacked higher molecular weight multimers as expected (Figure 2.3B; Waki *et al.*, 2003; Pajvani *et al.*, 2003). No high molecular weight species, expected in the range >300kDa (Waki *et al.*, 2003), were detected in the recombinant adiponectin, although they were detectable in mammalian adiponectin analysed using the same method (Figure 2.3C). Comparison of the bacterially expressed recombinant adiponectin and endogenous mammalian adiponectin shows the shift from high molecular weight to low molecular weight adiponectin that occurs in bacterial expression systems (Figure 3.3A and C). The mammalian trimer has a greater apparent size than its bacterial counterpart, possibly due to post-translational modifications such as glycosylation, that are not present in bacterial proteins, impeding the progression of the protein through the gel (Figure 2.3; Wang *et al.*, 2002). Also characterised was the breakdown of the recombinant adiponectin into apparent trimer, dimer and monomer species following reduction, heating and combined treatment respectively, to ensure that the recombinant protein was forming as expected (data not shown; Waki *et al.*, 2003).

2.3.3 Quantification of the recombinant adiponectin preparation

Quantification of adiponectin by ELISA confirmed that adiponectin was present at high levels in the recombinant protein preparations, with an average content of $1.7\pm0.4 mg.mL^{-1}$ adiponectin (Figure 2.4A). The increase in adiponectin content was reflected by increased total protein content (Figure 2.4B). Meanwhile, adiponectin detected in vehicle preparations was negligible, at $12.2\pm3.2 ng.mL^{-1}$ two orders of magnitude lower than that seen in the adiponectin samples (Figure 2.4A). However, the difference in total protein levels was much smaller, with vehicle preparations containing approximately a fifth the total protein observed in the adiponectin samples. This suggests that bacterial proteins are present, and are potentially also contaminating the adiponectin preparations despite not being apparent in the SDS-PAGE analysis.

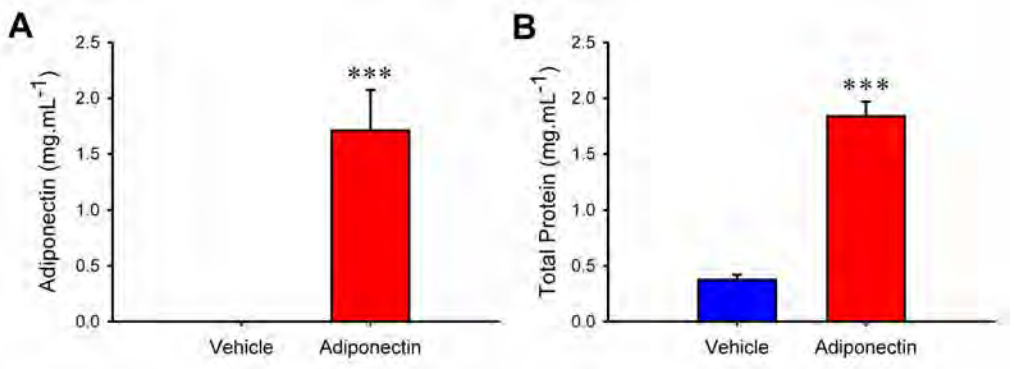

Figure 2.4: Quantification of adiponectin and total protein content of recombinant protein preparations. (A) Adiponectin content as determined by ELISA (*n*=5). (B) Total protein content as assayed using the Lowry method (*n*=9-11).

Comparison of the measured adiponectin and total protein content of the recombinant adiponectin and vehicle preparations would suggest that essentially all of the protein present in the adiponectin samples is adiponectin. This would in turn suggest that the vehicle contains a level of protein contamination that is not present in the adiponectin preparation, and thus may not be a genuine control. However, due to the extremely high levels of adiponectin present in the purified recombinant protein preparations, the samples had to be highly diluted to reach the range of the ELISA standard curve. Thus, there was a substantial degree of calculation required to achieve an estimate of the original adiponectin concentration. In addition, information regarding the affinity of this assay for the various adiponectin multimers is not available from the manufacturer. As the recombinant adiponectin consists primarily of LMW adiponectin multimers, whilst polyclonal antibodies directed against mammalian adiponectin were used in the assay, potential differential reactivity to the different adiponectin multimers may also affect the resulting values. Whilst these data clearly show significant enrichment of adiponectin in the recombinant protein samples, the absolute values may not provide an entirely accurate assessment of the exact adiponectin concentrations.

2.3.4 Adiponectin-stimulated eNOS phosphorylation

As the adiponectin produced for this study had not been used previously, it was deemed necessary to ensure that the recombinant adiponectin was biologically active. Adiponectin has previously been found to stimulate phosphorylation of eNOS in

endothelial cells *in vitro* (Chen *et al.*, 2003; Xi *et al.*, 2005). Thus, the extent of eNOS phosphorylation in response to the recombinant adiponectin was examined as an assessment of the bioactivity of the recombinant adiponectin produced in this study. Adiponectin dose-dependently increased phosphorylation of eNOS on serine 1177 in BAECs (Figure 2.5). This indicates that the recombinant adiponectin elicits biological activity similar to that reported in the literature.

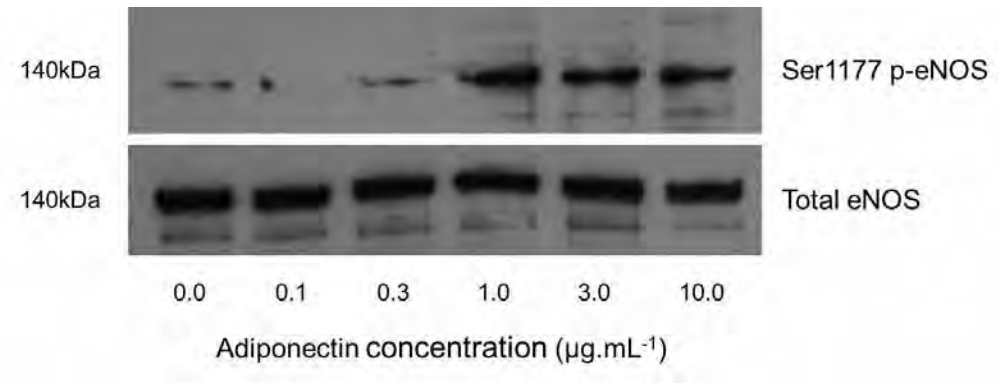

Figure 2.5: Phosphorylation of eNOS in response to adiponectin. Bovine aortic endothelial cells were treated with the indicated dose of adiponectin for 15min, before being immunoblot assayed for total and phosphorylated eNOS.

2.4 Discussion

The aim of this series of experiments was to produce purified, biologically active recombinant adiponectin. Murine full-length adiponectin was expressed in *E. coli* and purified by affinity chromatography. The preparation consisted of a single protein of the expected size of approximately 30kDa, as assessed by coomassie blue stained SDS-PAGE, indicating a relatively pure preparation. Under non-denaturing conditions, the full-length adiponectin preparation was shown to consist primarily of protein of approximately 70kDa, corresponding to a trimer, with small amounts of protein around 115-150kDa thought to represent mid-molecular weight multimers. Globular adiponectin was also produced, and identified as a single band at approximately 60kDa, consistent with a trimer, when immunoblotted under non-denaturing conditions. The purification procedure produced solutions sufficiently concentrated for physiological perfused rat hindlimb and *in vivo* experiments, containing approximately 1.7mg.mL^{-1} adiponectin. The full-length adiponectin was found to be biologically active, stimulating phosphorylation of eNOS in cultured endothelial cells. The protein profiles and biological activity shown here are similar to those reported in previous studies, indicating the protein produced was recombinant adiponectin.

The adiponectin produced in this study was expressed in bacteria, a system in which post-translational modifications are limited. Consequently, multimerisation of this adiponectin species is typically limited to LMW species. As described above and in Chapter 1, HMW adiponectin is likely to have a different range of actions to the LMW form. There is evidence that post-translational modifications may have additional roles unrelated to multimerisation, including a study using adiponectin containing a mutation of the cysteine residue required for disulphide bond formation. That protein more potently stimulated phosphorylation of AMPK and ACC in cultured myocytes than the wild-type recombinant protein expressed in bacteria, despite also being limited to a trimer (Waki *et al.*, 2003). Thus, the findings of investigations using the current adiponectin preparation can not be extrapolated to other forms of adiponectin.

It might be argued that the presence of a modified N-terminus could affect activity. However, preliminary experiments in cultured bovine aortic endothelial cells demonstrated dose-dependent stimulation of eNOS phosphorylation by the full-length adiponectin preparation, similar to that previously described (Hattori *et al.*, 2003; Cheng *et al.*, 2007). These positive results suggest that the tag was not significantly affecting the activity of the protein. Additionally, recombinant adiponectin with a similar attached N-terminal histidine tag was used in a study by Yamauchi *et al.* (2001), where adiponectin was found to ameliorate insulin resistance. These data suggest that removal of the tag is not required.

The recombinant adiponectin was cloned from murine cells, although it is intended for physiological experiments in rats. However, adiponectin protein sequences are highly conserved between species; the rat and mouse proteins are 91% conserved, and there is 83% similarity between mouse and human adiponectin (Nakano *et al.*, 1996). Most of the variation is within the N-terminal variable region, with the active globular domain and cysteine residues crucial for multimer formation particularly conserved (Tsao *et al.*, 2003). Many other studies have used divergent adiponectin preparations and test systems. Tomas *et al.* (2002) similarly used mouse adiponectin in isolated rat muscles to show increased fat oxidation and glucose uptake. In their study on adiponectin-mediated diminution of serotonin constriction, Fesus *et al.* (2007) used recombinant human adiponectin in rat aortic rings. Due to the high level of conservation of adiponectin proteins, the use of adiponectin from a different species should not affect experimental outcomes.

Any number of bacterial contaminants could potentially have persisted throughout the purification process. The discrepancy between the adiponectin and total protein estimates potentially suggests the presence of contaminating bacterial proteins. Proteins rich in histidine residues are particularly likely to be present in the final elution from the affinity column. Non-protein contaminants, including the endotoxin lipopolysaccharide, may also be present in the purified protein preparations. The presence of any bacterial contaminants may affect the results of physiological investigations. However, use of the vehicle control preparation should account for effects of the preparations that are not specific to adiponectin.

The aim of this series of experiments was to produce purified, biologically active recombinant adiponectin. The data shown here demonstrates that the adiponectin produced is of sufficient purity, quantity and structural integrity. The recombinant adiponectin displayed biological activity, to stimulate phosphorylation of eNOS, similar to previous reports. Thus it was concluded that these recombinant adiponectin preparations were suitable for use in physiological experiments.

CHAPTER 3 – ADIPONECTIN OPPOSES ENDOTHELIN-1-MEDIATED VASOCONSTRICTION IN THE PERFUSED RAT HINDLIMB.

3.1 Introduction

Previous studies in cultured cells and isolated vessels suggest that adiponectin is vasoactive. However, there has been little information as to whether this translates to any effects in an intact vascular system. The constant-flow pump-perfused rat hindlimb has been widely used to investigate skeletal muscle blood flow. This preparation consists of 95% skeletal muscle (Ruderman *et al.*, 1971), a tissue which accounts for over 85% of insulin-mediated glucose uptake (DeFronzo *et al.*, 1985). The perfused hindlimb also has the benefit of being devoid of circulating endogenous vasoactive factors and neural input, thus allowing experimental conditions to be tightly controlled. Therefore, the preparation allows for the acute examination of the direct effects of a physiological concentration of adiponectin, rather than augmenting endogenous levels. However, the perfused rat hindlimb is largely dilated under basal conditions, due its denervated state and the absence of endogenous regulatory factors. This means that dilatory responses are generally not apparent under basal conditions. For instance, insulin infusion alone causes no change in perfusion pressure in this system, despite its well described vasodilator actions *in vivo* (Clark *et al.*, 2003). The only reported hemodynamic effects of insulin in this preparation to date have been its ability to block endothelin-1 (ET-1)-mediated vasoconstriction (Kolka *et al.*, 2005) and a mild vasodilator response against norepinephrine (NE; Kolka *et al.*, 2005; Rattigan *et al.*, 1995). Thus, in this system, adiponectin-mediated vasodilation may only be detectable in the presence of vasoconstriction. Insulin-mediated vasodilation in the perfused hindlimb is primarily detectable during vasoconstriction due to ET-1, and the hemodynamic actions of adiponectin are hypothesised to be similar to those of insulin. Therefore, it is most likely that adiponectin-mediated vasodilation will be observed in the presence of ET-1-mediated vasoconstriction.

The endothelin family consists of three 21 amino acid peptides, with ET-1 considered the main isoform involved in the cardiovascular system (Plusczyk *et al.*, 2001; Levin, 1995). ET-1 is one of the most potent known vasoconstrictors, with a slow developing but sustained effect (Yanagisawa *et al.*, 1988). ET-1 is secreted abluminally by endothelial cells (Yoshimoto *et al.*, 1991) and elicits vasoconstriction predominantly via type A endothelin (ET_A) receptors situated on vascular smooth muscle cells (D'Orléans-Juste *et al.*, 1993; Maguire & Davenport, 1995). Circulating levels of ET-1 are increased in obese and type 2 diabetic subjects (Cardillo *et al.*, 2002; Ferri *et al.*, 1995; Takahashi *et al.*, 1990), and elevated plasma ET-1 is implicated in the development of hypertension and cardiovascular disease (Rich & McLaughlin, 2003; Shichiri *et al.*, 1990). It has been proposed that chronically elevated ET-1 leads to desensitisation of the insulin signalling pathway (Ishibashi *et al.*, 2001), and Wilkes *et al.* (2003) found that both acute and chronic infusion of ET-1 results in hyperinsulinemia and insulin resistance *in vivo*. Increased ET-1 in obesity and type 2 diabetes contributes to the endothelial dysfunction seen in these states, as defined by a reduced endothelium-dependent vasodilator response (Mather *et al.*, 2002). Inhibition of ET-1 activity via blockade of type A endothelin (ET_A) receptors restored NO-mediated endothelial function in apolipoprotein E-deficient mice (Barton *et al.*, 1998). ET-1 has also been shown to block the hemodynamic actions of insulin *in vivo*, along with the associated insulin-mediated glucose uptake (Ross *et al.*, 2007). Additionally, Shemyakin *et al.* (2010) have recently suggested that ET-1 may impair glucose uptake via a direct action on skeletal muscle cells. This bulk of evidence illustrates the multitude of detrimental effects resulting from uncontrolled ET-1 activity.

Adiponectin has been demonstrated to suppress ET-1-induced cellular hypertrophy in cultured cardiomyocytes (Fujioka *et al.*, 2006). I hypothesised that adiponectin may similarly be able to inhibit vasoconstriction caused by ET-1, via a NO-dependent vasodilator activity. Therefore, the aim of this study was to investigate the vascular actions of adiponectin in a perfused muscle system. After determining the effect of adiponectin alone on the vasculature, I investigated its effects on ET-1-mediated vasoconstriction. In the perfused hindlimb, at low doses ET-1 stimulates metabolism (Type A vasoconstriction) while high doses lead to metabolic inhibition (Type B vasoconstriction; Kolka *et al.*, 2005). These two different types of vasoconstriction are

thought to be the result of constriction occurring at different locations in the vascular tree, and thus affecting the extent of skeletal muscle perfusion (for review see Clark *et al.*, 1995). I then examined the action of adiponectin against low dose NE, a vasoconstrictor with similar dose-dependent metabolic actions to ET-1 in the perfused hindlimb (Dora *et al.*, 1992; Kolka *et al.*, 2005), to assess the specificity of any vascular action of adiponectin. I found that adiponectin specifically inhibits ET-1-mediated vasoconstriction by an apparently NO-independent mechanism.

3.2 Materials and Methods

3.2.1 Animals

All experiments were approved by the University of Tasmania Animal Ethics Committee and were performed in accordance with the guidelines set out under the NHMRC Code of Practice for the care and use of animals for scientific purposes seventh edition (2004). Male Hooded Wistar Rats (*n*=105) were housed at 21±1°C under a 12h light/dark cycle. Rats were provided with rat chow (Gibson's, Hobart, TAS, Australia), containing 21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fibre by weight, with added vitamins and minerals, and water *ad libitum*.

3.2.2 Perfused rat hindlimb

3.2.2.1 Perfusion Buffer

Krebs-Henseleit bicarbonate buffer (118mM NaCl, 4.7mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 25mM $NaHCO_3$) was supplemented with 8.3mM glucose and 4% (w/v) bovine serum albumin. This was passed through a 0.45µm filter under pressure and stored at -20°C for up to six months. Before use, the buffer was equilibrated at 32°C and gassed with 95% O_2: 5% CO_2 for 30min before the addition of 2.54mM $CaCl_2$, to avoid precipitation of calcium salts.

3.2.2.2 Surgery

Surgery was conducted on male 180-200g rats to isolate blood flow to a single hindlimb (Figure 3.1). The surgical procedures were essentially as described previously (Ruderman *et al.*, 1971), with additional details as outlined elsewhere (Colquhoun *et al.*, 1988).

Animals were anaesthetised with an intraperitoneal injection of pentobarbital sodium (50mg.kg^{-1} body weight; Ilium Veterinary Laboratories, Smithfield, NSW, Australia) and allowed to freely breathe room air. String ligatures were positioned around the tarsus of the experimental leg and the base of the tail to restrict blood flow. A ventral incision was made along the midline, through the skin and body wall. The inferior and

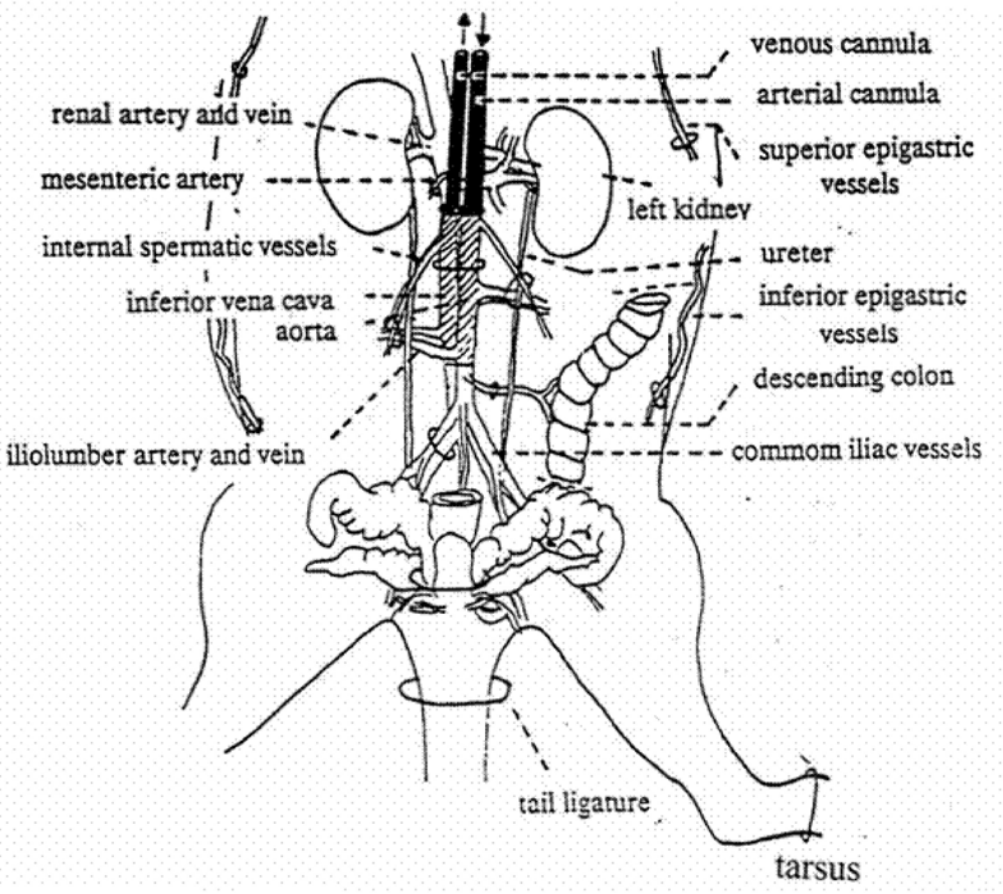

Figure 3.1: Perfused rat hindlimb surgery. Surgery details are given in section 3.2.2.2. Depicted is the ventral view of the rat, showing vessel ligation and cannulation performed to isolate blood flow to a single hindlimb (Adapted from Ross, 1972 and Greene, 1968).

superior epigastric arteries were ligated to allow removal of the body wall and greater access to the body cavity. The superficial epigastric artery of the experimental leg was ligated adjacent to the femoral vessels to restrict flow to the skin of the hindlimb. The testes were tied off and removed, and a single ligature was placed around the bladder and seminal vesicles and the seminal vesicles were removed. Two ties were placed around the lower descending colon approximately 1cm apart, and the colon was cut between these ties. A further tie was placed around the duodenum and the superior mesenteric artery just below the stomach, and the gastrointestinal tract was removed. The connective tissue was gently separated, allowing the ureter, internal spermatic vessels and iliolumbar vessels on both sides to be tied off together. The common iliac artery supplying the contra-lateral hindlimb was also ligated, as only one hindlimb was perfused.

The inferior vena cava and descending aorta were separated and two silk threads were loosely positioned around each one between the origins of the renal and iliolumbar vessels. Heparin (200IU in 200µL) was injected into the vena cava above the renal vessel. The vena cava was ligated below the renal vessel and an 18G, 1¼ inch Surflo® cannula (Terumo, Elkton, MD, USA) was inserted and secured in place with the lower tie. The aorta was cannulated in the same manner, using a small cut with fine scissors and a needle threader to facilitate insertion of a 20G, 1¼ inch Surflo® cannula (Terumo, Elkton, MD, USA). The cannulae were connected to the perfusion line to provide perfusion buffer to the hindlimb. Once the hindlimb was connected to the perfusion network, the animal was sacrificed with an intracardiac overdose of pentobarbital sodium. Denervation occurs through loss of central nervous system function following sacrifice. A further string ligature was positioned around the abdomen at approximately the level of the L3 vertebra to impede extraneous flow. The entire procedure was completed within 30min, with care taken to ensure perfusion of the experimental hindlimb was interrupted for no more than 2min.

3.2.2.3 Perfusion apparatus

Hindlimbs were perfused in a non-recirculating manner with perfusion buffer, using a Masterflex® peristaltic pump (Cole-Parmer, Vernon Hills, IL, USA) to ensure a constant flow rate of 5mL.min^{-1}. The perfusate was gassed with 95% O_2: 5% CO_2 in a silastic tube oxygenator. Experiments were conducted at 32°C in a temperature controlled cabinet, with the buffer temperature maintained by passage through a water-jacketed heat exchange coil. Test substances were infused into a small magnetically stirred bubble trap located in the arterial perfusion line via a programmable syringe pump (World Precision Instruments, Sarasota, FL, USA). Perfusion pressure was constantly monitored via a pressure transducer located in the arterial line. Venous effluent oxygen content was measured using a 0.5mL flow-through Clark-type oxygen electrode assembly, also maintained at 32°C. The venous effluent was periodically sampled and the remainder discarded. Perfusion pressure and oxygen content was continually recorded using WinDaq data acquisition software (Dataq Instruments, Akron, OH, USA). This apparatus is depicted in Figure 3.2.

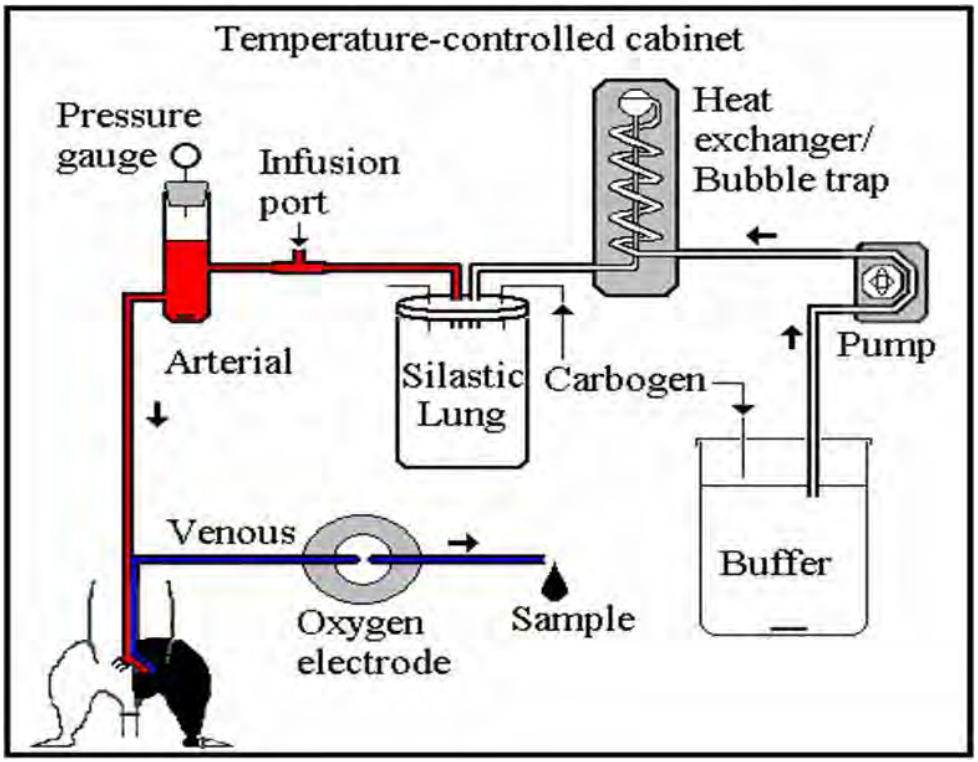

Figure 3.2: Apparatus for the non-recirculating constant flow, pump-perfused rat hindlimb. The apparatus is described in section 3.2.2.3. (Taken from Kolka, 2006).

3.2.2.4 Perfusion protocols

The hindlimb was allowed to equilibrate for 30-40min in order to reach a steady-state before any experimental protocols were commenced. Venous effluent was sampled for glucose and lactate determinations every five minutes. All test substances were dissolved in 0.9% NaCl before infusion. Insulin (Humulin R; Eli Lilly, Indianapolis, IN, USA) was diluted 1:200 in saline and infused at 25µL.min^{-1}, to provide 15nM to the hindlimb. ET-1 (Calbiochem, San Diego, CA, USA) was dissolved in acetic acid and made up to volume in saline. NE (Arterenol) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Vasoconstrictors were infused at 25µL.min^{-1}, 1/200th of the pump flow rate, providing final concentrations of 1nM or 20nM ET-1 and 50nM NE. Adiponectin solutions were diluted to 0.65mg.mL^{-1} in saline and infused at 50µL.min^{-1}, equivalent to 1/100th of the pump flow rate, to give a final concentration of 6.5µg.mL^{-1}. This dose was selected to mimic normal circulating levels of adiponectin in rats, approximately 6µg.mL^{-1} under normal conditions (Satoh *et al.*, 2005). Globular adiponectin was diluted to 0.46mg.mL^{-1} in saline and infused in the same way as the full-length form to provide an equimolar final concentration. The vehicle

solutions were diluted to the same extent as the adiponectin solutions, so that the amount of contaminants, but not total protein content, remained consistent. Individual protocols are shown in Figure 3.3.

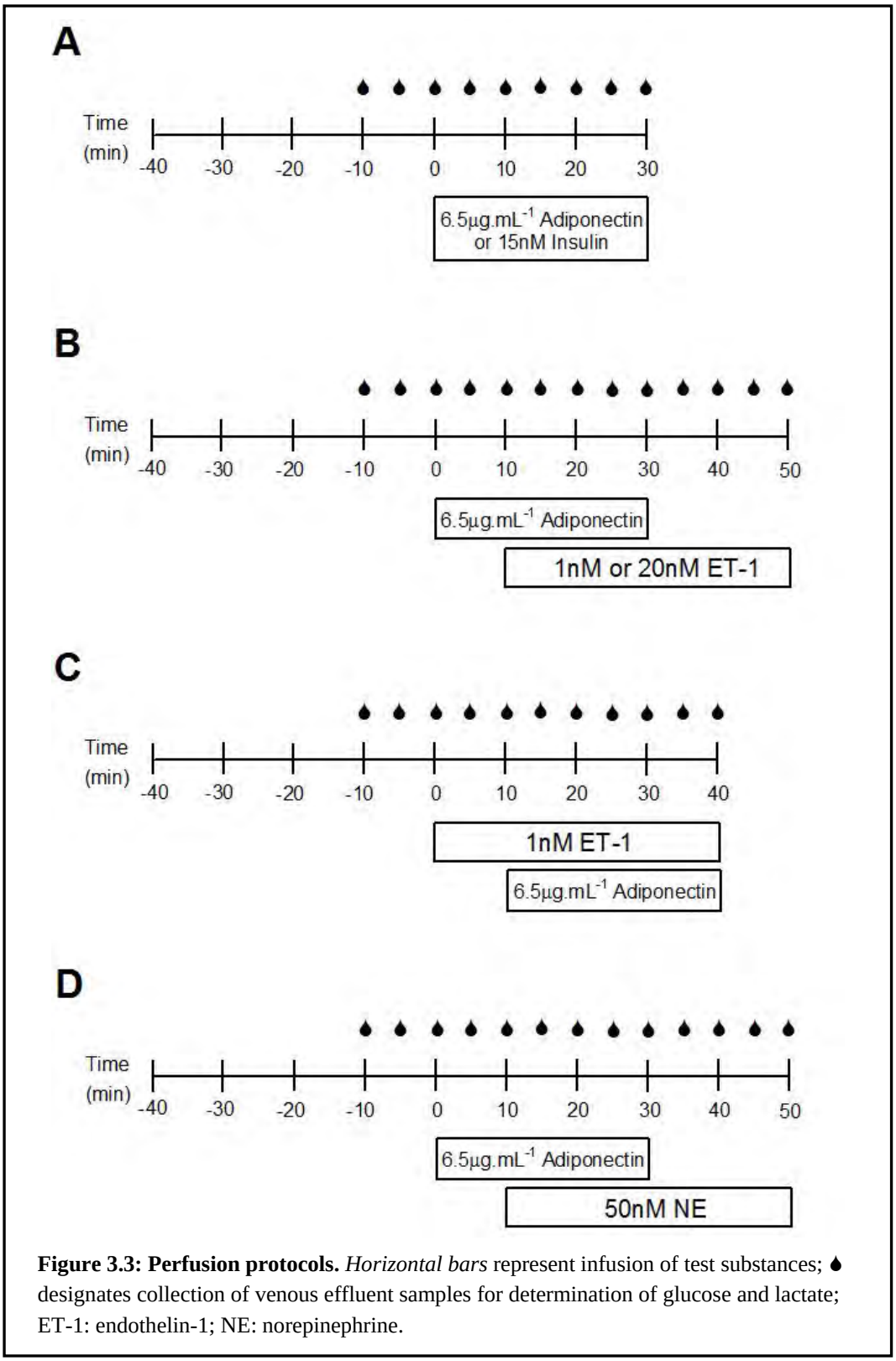

Figure 3.3: Perfusion protocols. *Horizontal bars* represent infusion of test substances; ♦ designates collection of venous effluent samples for determination of glucose and lactate; ET-1: endothelin-1; NE: norepinephrine.

3.2.2.5 Measurement of glucose and lactate

Glucose and lactate determinations were made using a glucose analyser (Model 2300 Stat Plus, Yellow Springs Instruments), and hindlimb uptake or release data respectively were derived from the product of flow, arterio-venous (A-V) concentration difference and perfused muscle mass.

3.2.2.6 Calculation of oxygen consumption

The oxygen electrode was calibrated with air and oxygen, and the oxygen content of the arterial perfusion buffer was measured before and after each experiment.

Muscle oxygen consumption was calculated using the Fick principle:

$$VO_2 = \frac{\beta \times (P_aO_2 - P_vO_2) \times (flow/1000) \times 60}{\text{muscle weight (g)}}$$

* Where β = calculated from the Bunsen coefficient

$\beta = \alpha / (22.4 \times 760)$

$= 0.0230 mL.L^{-1} / (22.4 mM \times 760 mmHg)$

$= 1.351 \mu mol.L^{-1}.mmHg$

(α = the volume (mL) of oxygen dissolved per mL of plasma at 0°C and 760mmHg. α is 0.0230 in plasma at 32°C.)

* Where P_aO_2 = arterial PO_2 using the arterial oxygen measurement and the air and oxygen calibrations from the oxygen electrode, and using their known PO_2.

$$P_aO_2 = \frac{\text{Art} - \text{cal Air}}{\text{cal } 100\%O_2 - \text{cal Air}} \times (PO_2 \text{ at } 100\% - PO_2 \text{ in Air}) + PO_2 \text{ in Air}$$

where: Art = electrode arterial measurement

cal Air = electrode air calibration

cal $100\%O_2$ = electrode oxygen calibration

PO_2 at 100% = 760mmHg – 36mmHg (H_2O vapour pressure at 32°C, due to the use of a wet oxygen electrode)

= 724mmHg

PO_2 in Air = 154mmHg

* Where P_vO_2 = venous PO_2 calculated the same as the P_aO_2, however the value for Art was replaced by the value measured in the venous effluent.

* Where flow = perfusion flow rate in $mL.min^{-1}$

* Where muscle weight = total perfused muscle mass, which has previously been determined to be 8.3% of the body weight for a single perfused hindlimb (Richter *et al.*, 1982).

3.2.3 Statistics

Two-way repeated measures ANOVA was performed using SigmaStat (SPSS Science, Chicago, IL, USA). Reversal of ET-1 effects (section 3.3.2.3) was assessed using a one way ANOVA. Differences between conditions were assessed using the Student-Neuman-Keuls post hoc test. Significance was assumed at the level of $p<0.05$. Data are presented as means ± standard error; if error bars are not visible, they are within the symbol.

3.3 Results

3.3.1 Hemodynamic and metabolic actions of full-length adiponectin

Under basal conditions at a flow rate of 5mL.min^{-1}, average measurements were perfusion pressure: 28.7±3.3mmHg, oxygen uptake: 10.6±1.2µmol.g^{-1}.h^{-1}, glucose uptake: 9.0±1.0µmol.g^{-1}.h^{-1}, and lactate release: 20.6±2.4µmol.g^{-1}.h^{-1} (n=74). Infusion of full-length adiponectin alone did not elicit any change in perfusion pressure, indicating that full-length adiponectin does not have any hemodynamic effects under basal conditions, in the absence of endogenous vasoconstriction (Figure 3.4A). Full-length adiponectin caused a significant stimulation of hindlimb glucose uptake (p<0.05), although this effect was much smaller than that seen with insulin (adiponectin 1.58±0.21 vs. insulin 2.47±0.12 fold increase from basal; p<0.01; Figure 3.4C). Glucose uptake levels returned to basal within 5min of the full-length adiponectin infusion ceasing, indicating that this is a relatively rapid and reversible

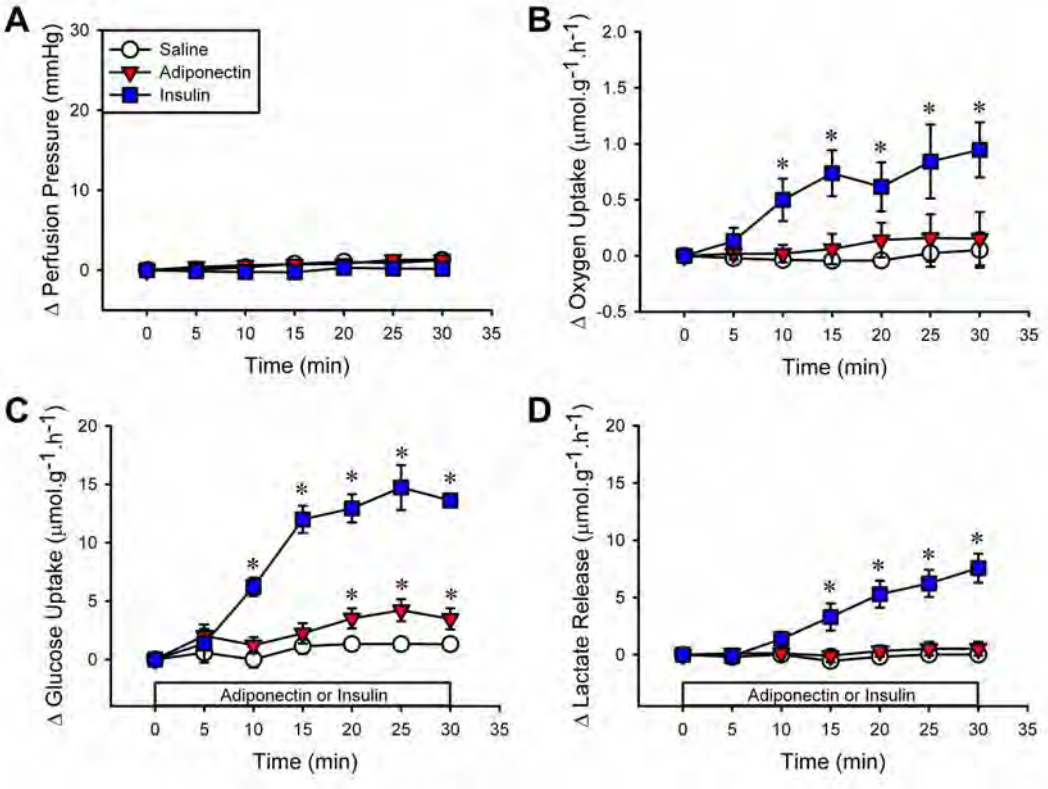

Figure 3.4. Effect of adiponectin or insulin on (A) perfusion pressure, (B) oxygen uptake, (C) glucose uptake and (D) lactate release. Substances were infused at the times indicated according to Figure 3.3A. Data are normalised to t=0min according to Figure 3.3A. * Significantly different from saline (p<0.05; n=9-10 except insulin n=3).

effect (data not shown). Despite the observed increase in glucose uptake during full-length adiponectin infusion, there was no concomitant increase in lactate release, unlike that seen with insulin treatment, suggesting that the transported glucose is either oxidised or stored as glycogen (Figure 3.4D). Additionally, no significant adiponectin-induced change in oxygen consumption was seen under basal conditions (Figure 3.4B).

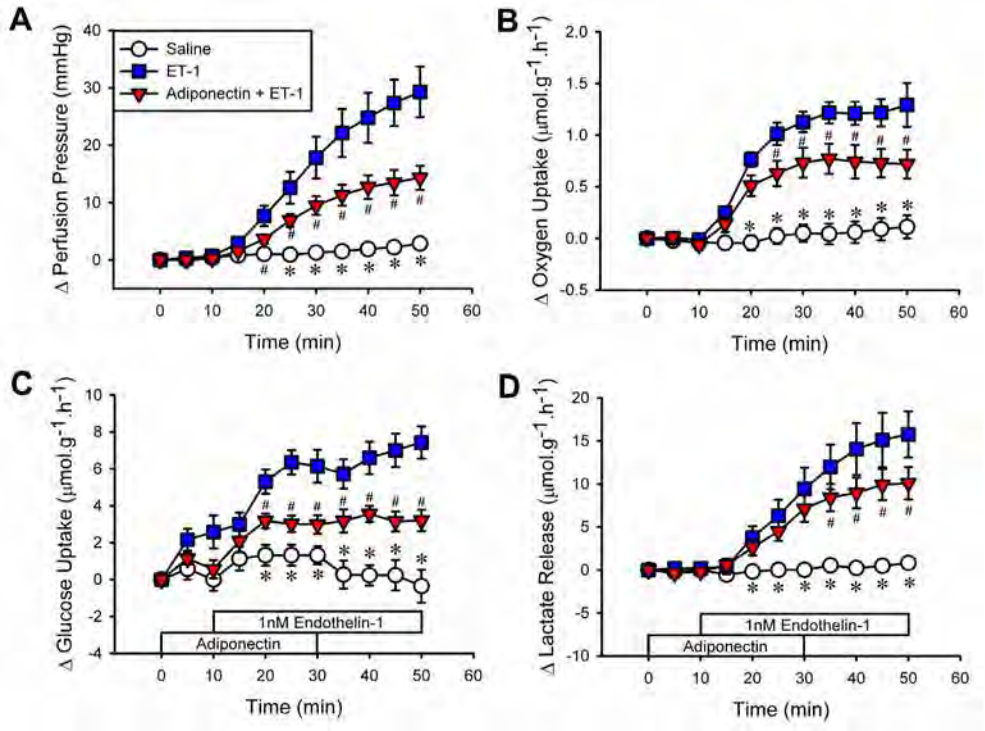

Figure 3.5: Effect of adiponectin on ET-1-mediated changes in (A) perfusion pressure, (B) oxygen uptake, (C) glucose uptake and (D) lactate release. Substances were infused at the times indicated according to Figure 3.3B. Data are normalised to *t*=0min. * Significantly different from all other groups and # significantly different from ET-1 alone ($p<0.05$; n=8-10).

3.3.2 Effect of adiponectin on the hemodynamic and metabolic effects of endothelin-1

3.3.2.1 Adiponectin opposes vasoconstriction caused by low dose endothelin-1

Low-dose ET-1 (1nM) significantly increased perfusion pressure within 10min of administration, with an increase of 29.3±4.4mmHg following a 40min infusion ($p<0.001$; Figure 3.5A). The increased perfusion pressure was accompanied by the enhanced metabolism typical of Type A vasoconstrictors (Clark *et al.*, 1995), indicated by increases in oxygen consumption (1.3±0.2μmol.g^{-1}.h^{-1}), glucose uptake

(7.4±0.9μmol.g^{-1}.h^{-1}), and lactate release (15.8±2.7μmol.g^{-1}.h^{-1}) after 40min, similar to the effects that have previously been reported (Kolka *et al.*, 2005). Pre-treatment and co-infusion with physiological levels of full-length adiponectin led to a significant attenuation of ET-1-mediated vasoconstriction from 15min after ET-1 infusion commenced until the end of the protocol (Figure 3.5A). Perfusion pressure was increased only by 14.3±2.1mmHg representing a 51% reduction compared to ET-1 alone (p<0.001). In conjunction, the associated metabolic stimulation was also diminished, with the increases in oxygen consumption, glucose uptake and lactate release blunted by 44% (0.7±0.1μmol.g^{-1}.h^{-1}), 57% (3.2±0.6μmol.g^{-1}.h^{-1}) and 36% (10.0±1.9μmol.g^{-1}.h^{-1}) respectively (Figure 3.5). The attenuated response to ET-1 was maintained after adiponectin co-infusion ceased, and indeed the differences between the two groups continued to increase for the duration of the experiment, indicating that the vascular actions of adiponectin are not rapidly reversible. A vehicle preparation, isolated by the same process used for adiponectin, but from untransformed bacteria, had no effect on the any of the actions of ET-1 measured (Figure 3.6) indicating that the observed effects were specific to adiponectin and not the result of nonspecific contaminants.

The effect of a preparation of the truncated, globular form of adiponectin on ET-1-mediated vasoconstriction was evaluated to determine whether globular adiponectin acts more potently than the full-length trimer in the skeletal muscle vasculature. ET-1-mediated perfusion pressure development was reduced by 49% by pre-treatment and co-infusion of globular adiponectin (11.2±1.7mmHg vs. 21.7±1.0mmHg with ET-1 alone; Figure 3.7A). Similarly, globular adiponectin decreased ET-1-stimulated oxygen consumption by 58% (0.6±0.4μmol.g^{-1}.h^{-1} vs. 1.6±0.1μmol.g^{-1}.h^{-1} with ET-1 alone; Figure 3.7B). ET-1-mediated lactate release was abolished by infusion of globular adiponectin, with lactate maintained at basal levels over 40min of ET-1 infusion (2.2±0.9μmol.g^{-1}.h^{-1} vs. 13.1±0.3μmol.g^{-1}.h^{-1} with ET-1 alone; p<0.001). Globular adiponectin did not affect the glucose uptake stimulated by ET-1 (8.1±3.6μmol.g^{-1}.h^{-1} vs. 9.7±3.2μmol.g^{-1}.h^{-1} with ET-1 alone at t=50min; NS). The lack of an inhibitory effect of globular adiponectin on ET-1-mediated glucose uptake may be due to confounding with an effect of globular adiponectin itself on glucose uptake. These data showed that the globular adiponectin was not markedly more

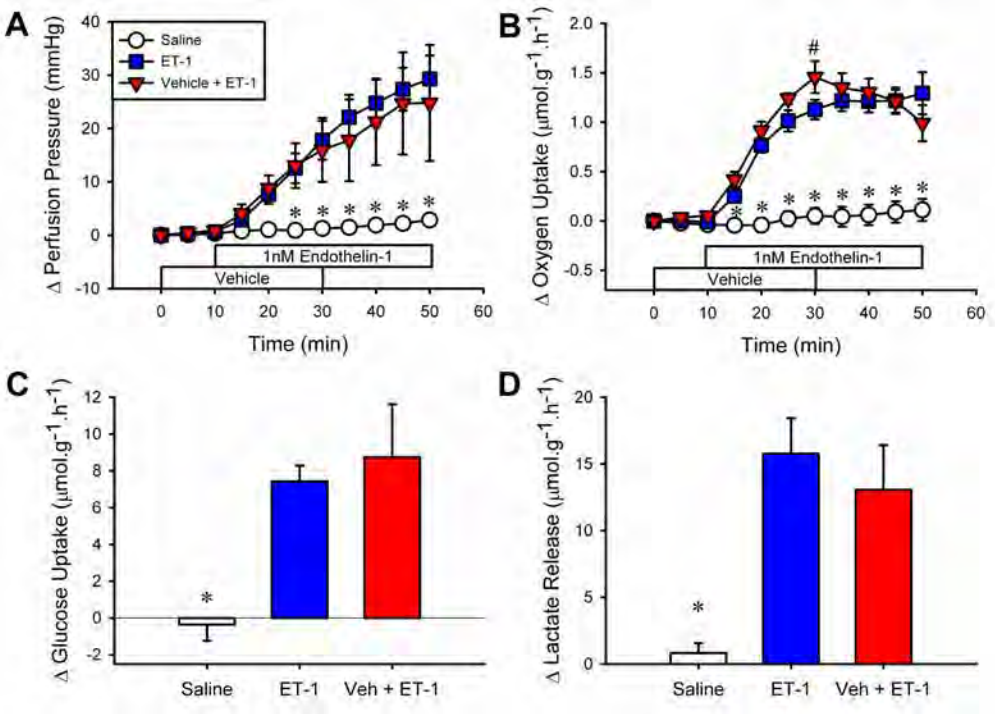

Figure 3.6: Effect of vehicle on ET-1-mediated changes in (A) perfusion pressure, (B) oxygen uptake, (C) glucose uptake at *t*=50min and (D) lactate release at *t*=50min. Substances were infused at the times indicated according to Figure 3.3B. Data are normalised to *t*=0min. Veh: Vehicle. * Significantly different from all other groups and # significantly different from ET-1 alone ($p<0.05$; n=8-10 except vehicle n=3).

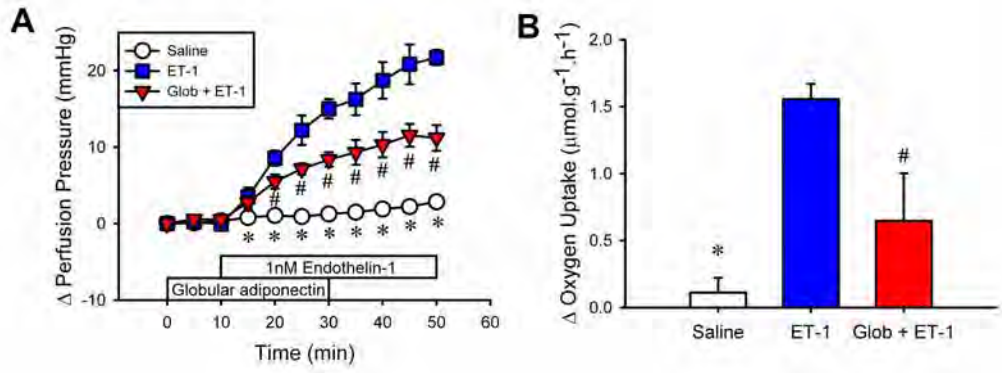

Figure 3.7: Effect of 4.6μg.mL^{-1} globular adiponectin on ET-1-mediated changes in (A) perfusion pressure and (B) oxygen uptake at *t*=50min. Substances were infused at the times indicated according to Figure 3.3B. Data are normalised to *t*=0min. Glob: globular adiponectin. * Significantly different from all other groups and # significantly different from ET-1 alone ($p<0.05$; n=3 except saline n=10).

potent, but had similar vascular effects to the full-length protein, with both preparations approximately halving the ET-1 response. Therefore, globular and LMW

adiponectin have comparable vascular activity. All further investigations were thus conducted using only the full-length adiponectin preparation.

3.3.2.2 Adiponectin is ineffective against established endothelin-1-mediated vasoconstriction

I next examined whether adiponectin was able to oppose established ET-1-mediated vasoconstriction. The previous experiments were repeated with the adiponectin infusion commencing 10min after commencement of ET-1 infusion. Once ET-1 vasoconstriction had developed, a physiological dose of adiponectin was unable to attenuate the increase in perfusion pressure or the associated stimulation of oxygen uptake (Figure 3.8). Similarly, there was no significant difference in glucose uptake between rats treated with adiponectin and those without, with an increase of $5.5\pm1.2\mu mol.g^{-1}.h^{-1}$ following co-infusion compared to $4.8\pm0.8\mu mol.g^{-1}.h^{-1}$ after 40min of ET-1 alone (NS). Adiponectin did cause a small but significant diminution in lactate release by the end of the experiment, reducing it to $11.6\pm1.4\mu mol.g^{-1}.h^{-1}$ compared to $15.6\pm2.4\mu mol.g^{-1}.h^{-1}$ after ET-1 infusion alone ($p<0.05$).

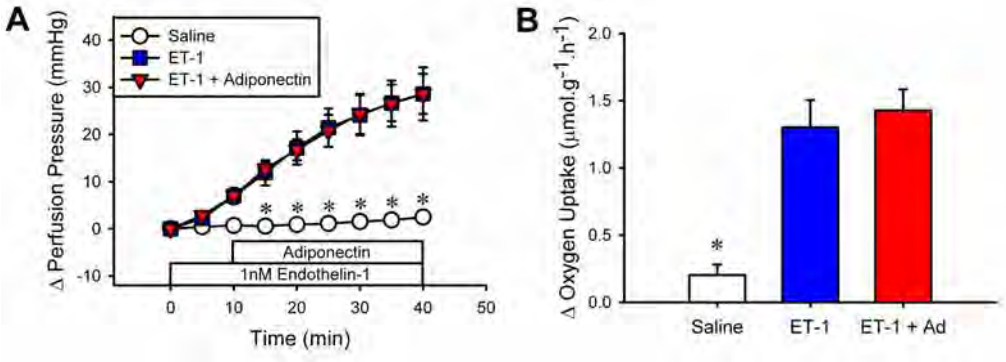

Figure 3.8: Effect of adiponectin on ET-1-mediated changes in (A) perfusion pressure and (B) oxygen uptake at t=40min. Substances were infused at the times indicated according to Figure 3.3C. Data are normalised to *t*=0min. Ad: Adiponectin. * Significantly different from all other groups ($p<0.05$; n=7-10).

3.3.2.3 Low-dose endothelin-1 vasoconstriction is reversible

The inability of adiponectin to oppose ET-1-mediated vasoconstriction once it was established suggested that adiponectin was interfering in ET-1 signalling, and not directly binding ET-1. However, ET-1-mediated vasoconstriction is long-lasting, with one study showing that human forearm blood flow did not fully recover until 120min after cessation of a 60min infusion of ET-1 (Clarke *et al.*, 1989). Therefore,

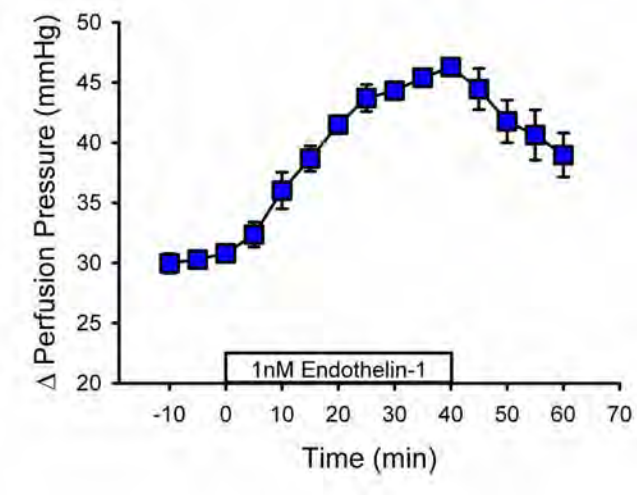

Figure 3.9: ET-1-induced perfusion pressure is rapidly reversed upon cessation of ET-1 infusion. ET-1 was infused over the time indicated. (*n*=2).

adiponectin-mediated reversal of ET-1 vasoconstriction may not be observable with the current 30min infusion. To address this, I determined the reversibility of ET-1-mediated vasoconstriction in the perfused rat hindlimb. A 40min infusion of ET-1 alone was undertaken as in the previous experiments, and at the end of this period the ET-1 infusion was stopped while monitoring continued for a further 20min. Twenty minutes after the ET-1 infusion was stopped, the perfusion pressure had decreased 45% from the peak ET-1-mediated increase in perfusion pressure (change 9.0±2.7mmHg at 60min vs. 16.3±1.1mmHg at 40min; $p<0.05$; Figure 3.9). There was also a trend for reduced lactate release 20min post ET-1 infusion, with a decrease of 57% from the peak (change 6.0±1.7μmol.g^{-1}.h^{-1} at 60min vs. 13.8±3.1μmol.g^{-1}.h^{-1} at 40min; $p=0.074$). However, 20min after the ET-1 infusion ceased, there was no apparent change in either oxygen uptake (change 0.8±0.3μmol.g^{-1}.h^{-1} at 60min vs. 0.9±0.3μmol.g^{-1}.h^{-1} at 40min) or glucose uptake (change 3.5±3.1μmol.g^{-1}.h^{-1} at 60min vs. 4.3±2.6mol.g^{-1}.h^{-1} at 40min), indicating that these effects of ET-1 are more sustained. Whilst the effects of ET-1 are far more sustained than most other vasoconstrictors, many of which reverse within 5min of infusion withdrawal, ET-1-mediated vasoconstriction is diminished 10-20min after it is removed. Thus, if adiponectin were effective at reducing established ET-1-mediated vasoconstriction, this should be observable within the 30min adiponectin infusion used in the present study.

3.3.2.4 Adiponectin is unable to oppose high dose endothelin-1

The biphasic dose-response to ET-1 that has previously been reported was again demonstrated (Kolka *et al.*, 2005). However, the high ET-1 dose (10nM) used previously was not sufficient to produce net metabolic inhibition in any of the parameters measured at the lower flow rate used in the current study. Therefore, the ET-1 dose was increased to 20nM to produce a type B vasoconstrictor response.

A 20nM ET-1 infusion elicited a marked increase in perfusion pressure of 185.8±13.7mmHg (p<0.001; Figure 3.10A), but unlike the 1nM ET-1 response, the high dose caused a transient metabolic stimulation, before shifting towards an inhibition of metabolism. After a 40min infusion of high-dose ET-1, oxygen consumption was significantly inhibited compared to basal, with a net decrease in oxygen uptake of 2.9±0.7µmol.g^{-1}.h^{-1} (p<0.001; Figure 3.10B). Glucose uptake and lactate release showed a similar trend, with significant (p<0.001) increases of 10.6±0.8µmol.g^{-1}.h^{-1} and 40.2±3.0µmol.g^{-1}.h^{-1} respectively after 10min of ET-1 infusion, and a return to near basal levels by 40min (Figure 3.10). Thus, high doses of ET-1 were again seen to display a Type B vasoconstrictor response with an overall

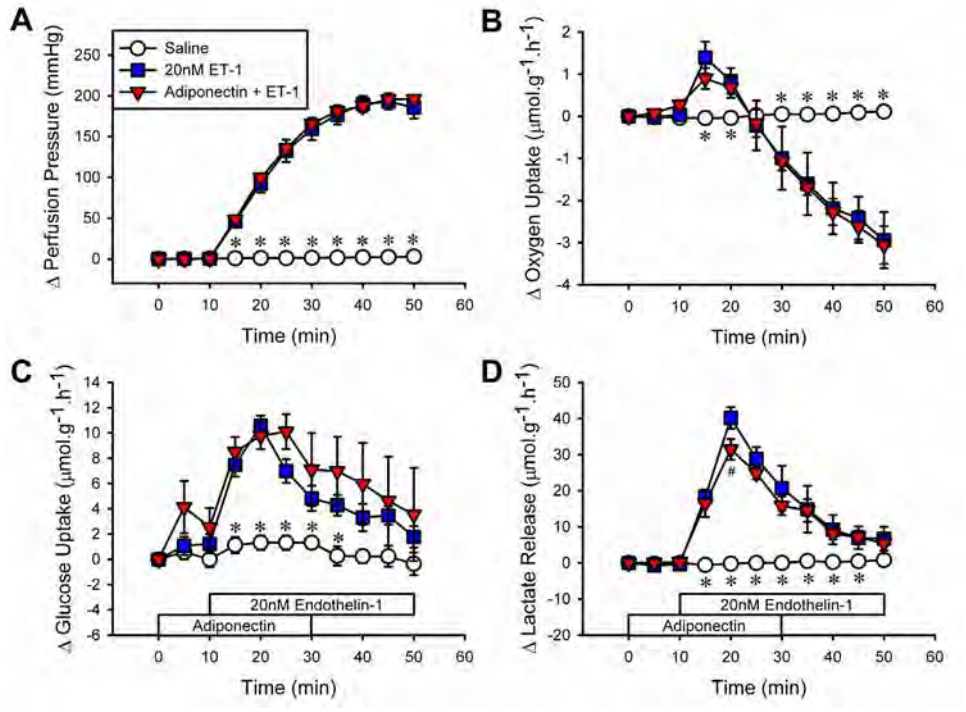

Figure 3.10: Effect of adiponectin on high-dose ET-1-mediated changes in (A) perfusion pressure, (B) oxygen uptake, (C) glucose uptake and (D) lactate release. Substances were infused at the times indicated according to Figure 3.3B. Data are normalised to t=0min. * Significantly different from all other groups and # significantly different from ET-1 alone (p<0.05; n=6-10).

metabolic inhibition (Clark *et al.*, 1995). There was no overall significant difference in any of the parameters measured between rats treated with 20nM ET-1 alone or in conjunction with adiponectin. Therefore, physiological adiponectin was unable to overcome the effects of high-dose ET-1-mediated vasoconstriction (Figure 3.10).

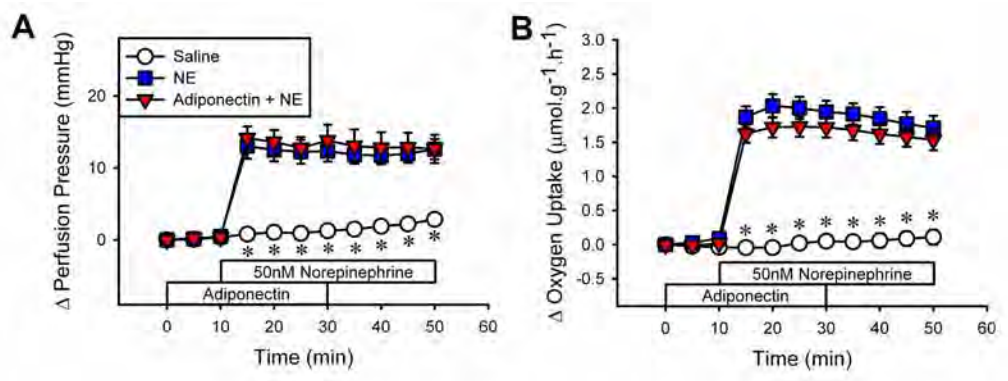

Figure 3.11: Effect of adiponectin on NE-mediated changes in (A) perfusion pressure and (B) oxygen uptake. Substances were infused at the times indicated according to Figure 3.3D. Data are normalised to *t*=0min. * Significantly different from all other groups ($p<0.05$; n=8-10).

3.3.3 Adiponectin does not effect the hemodynamic and metabolic actions of norepinephrine

Infusion of low dose NE caused rapid vasoconstriction, demonstrated by an increase in perfusion pressure of 13.0mmHg after 5min ($p<0.001$; Figure 3.11A). A low NE dose was used to stimulate Type A vasoconstriction (Clark *et al.*, 1995), similar to that observed with low-dose ET-1. Enhanced metabolism was demonstrated by a significant increase in oxygen consumption of 1.7±0.2μmol.g^{-1}.h^{-1} after 40min of treatment ($p<0.001$ vs. saline; Figure 3.11B). Glucose uptake and lactate release were similarly stimulated, with maximal increases of 5.8±0.9μmol.g^{-1}.h^{-1} and 8.2±1.4μmol.g^{-1}.h^{-1} respectively after 5min of NE infusion ($p<0.001$ vs. saline). Pre-treatment and co-infusion with adiponectin, using the same protocol as the original ET-1 experiments, had no effect on any measure of NE action under these conditions (Figure 3.11).

3.4 Discussion

This study demonstrated that adiponectin is vasoactive in an intact perfused muscle system, with the ability to restrict ET-1-mediated vasoconstriction. Adiponectin alone, however, had no observable hemodynamic actions in this system. Adiponectin was unable to overcome vasoconstriction due to ET-1 once it had developed, or that caused by high doses of ET-1. Additionally, under the same conditions, adiponectin infusion had no effect on NE-induced vasoconstriction, suggesting adiponectin does not act as a general vasodilator.

The attenuation of ET-1 action by adiponectin observed in this study appears to be due to a specific action of adiponectin on ET-1-mediated vasoconstriction and its associated metabolic effects. Full-length LMW adiponectin and globular adiponectin were shown to have similar effects on ET-1 action. No hemodynamic changes were observed during the infusion of adiponectin alone, as is the case with insulin. Additionally, in both previous reports of insulin attenuating vasoconstriction it was found to be ineffective once vasoconstriction is established (Kolka *et al.*, 2005; Rattigan *et al.*, 1995). Thus, the effects of adiponectin reported here appear to be similar to those previously described for insulin, which does not have a general vasodilatory activity against all vasoconstrictors in the perfused rat hindlimb. Given the reported insulin sensitising effects of adiponectin, there is also potential for greater inhibition of ET-1 in the presence of insulin, and such a relationship warrants further investigation. Adiponectin was unable to overcome vasoconstriction due to high-dose ET-1, potentially suggesting an adiponectin resistance situation in hypertensive patients where ET-1 levels are elevated. However, it is unknown whether *in vivo* ET-1 reaches the levels I have used in the perfused hindlimb, with the doses used in this study several orders of magnitude higher than reported ET-1 plasma concentrations (Shichiri *et al.*, 1990; Takahashi *et al.*, 1990). However, the local nature of ET-1 production means that the possibility of localised high concentrations cannot be excluded.

The inability of adiponectin to moderate the actions of ET-1 when introduced after vasoconstriction is established suggests that adiponectin may not, in fact, be acting as a NO-dependent vasodilator as hypothesised. Such a general vasodilatory action would be expected to reduce vasoconstriction regardless of prior pressure development, given that ET-1 vasoconstriction was reversible within the timeframe used. In contrast to adiponectin, SNP, a direct NO donor, is able to rapidly reduce perfusion pressure when administered during ET-1-mediated vasoconstriction (Kolka, 2006). Chen *et al.* (2003) studied adiponectin-mediated NO production in endothelial cells and found that this effect was rapid, reaching its peak within a few minutes of exposure to adiponectin. This suggests that were adiponectin-mediated inhibition of ET-1 occurring via endothelial NO release, it would be a rapid effect and thus not require pre-treatment as demonstrated in the present study. The finding that adiponectin did not diminish NE-mediated vasoconstriction, while the endothelial NO-dependent dilators UTP and ATP both rapidly dilate against NE (Richards *et al.*, 1993), further points to a NO-independent process. In fact, there is a precedent for this in a recent study by Fesus *et al.* (2007), who reported adiponectin-mediated relaxation of de-endotheliated rat aortic rings pre-constricted with serotonin. Additionally, insulin has been shown to have dual actions, the well characterised NO-dependent effect, but also a direct effect on smooth muscle to reduce agonist-induced increases in intracellular calcium concentration ($[Ca^{2+}]_i$; Han *et al.*, 1995). This further supports the possibility of a NO-independent mechanism of adiponectin action, as well as providing a potential explanation for the specific relationship both insulin and adiponectin have with ET-1. Adiponectin-mediated inhibition of ET-1-mediated vasoconstriction is maintained after adiponectin is removed from this constant flow-through system, whilst the ET-1 supply remains. Additionally, adiponectin was unable to reverse ET-1-mediated vasoconstriction once it was established, although the vasoconstriction was demonstrated to be reversible. Therefore, attenuation of ET-1 action by adiponectin is unlikely to be due to either direct binding of adiponectin to ET-1 or adiponectin interfering with the interaction between ET-1 and its ET_A receptor. Thus, it is possible that the hemodynamic effects of adiponectin described here may be due to a specific inhibitory action on the ET-1 signalling pathway in vascular smooth muscle cells.

Endothelin-1 binds to the G-protein coupled ET_A receptor on vascular smooth muscle cells (VSMC), stimulating the production of inositol trisphosphate (IP_3), which in turn stimulates the release of calcium (Ca^{2+}) from the sarcoplasmic reticulum (SR), causing a transient peak in $[Ca^{2+}]_i$ (Pollock *et al.*, 1995). ET-1 can sustain this increase in $[Ca^{2+}]_i$ by stimulating extracellular Ca^{2+} entry via non-selective cation channels and store-operated Ca^{2+} channels (Furutani *et al.*, 2002). Raised $[Ca^{2+}]_i$ causes contraction of VSMC, resulting in vasoconstriction. NE elicits vasoconstriction via similar mechanisms, probably involving both IP_3-dependent mobilisation of Ca^{2+} from SR and Ca^{2+} entry via L-type voltage-gated channels, followed by store-operated capacitative entry (Webb, 2003; Furutani *et al.*, 2002). Therefore, the considerable overlap between the ET-1 and NE contractile pathways leaves few targets for the specific action of adiponectin, although differences in the ion channels activated for extracellular calcium entry, such as the L-type voltage-operated calcium channels reported to be activated by NE but not ET-1 (Furutani *et al.*, 2002), may provide an avenue for investigation. Alternatively, the requirement for adiponectin to be present prior to ET-1 exposure suggests a mechanism early in the pathway; probably operating between the receptor and IP_3 formation, before the signalling pathways of the two vasoconstrictors merge. It is tempting to hypothesise that by inhibiting the initial IP_3-mediated Ca^{2+} release from SR, adiponectin may be able to prevent the sustained rise in $[Ca^{2+}]_i$ and therefore block ET-1-mediated vasoconstriction. However, conflicting reports concerning whether blocking the peak $[Ca^{2+}]_i$ response to ET-1 actually prevents the sustained increase in $[Ca^{2+}]_i$ casts doubt on this theory (Thai *et al.*, 2009; Wagner-Mann & Sturek, 1991). Bergdahl *et al.* (2003) found that cholesterol depletion in endothelium-denuded caudal arteries reduced contractility to ET-1 by disrupting the association between the ET_A receptor and transient receptor potential channel 1 (TRPC1) within caveolae. The ET-1 response was affected following a 20 min pre-treatment with the cholesterol acceptor methyl-β-cyclodextrin (mβcd), but not when ET-1 and mβcd were added simultaneously (Bergdahl *et al.*, 2003). In addition, cholesterol depletion had no such effect on α_1-receptor stimulation (Dreja *et al.*, 2002), such as that elicited by NE. This profile closely matches the action of adiponectin described here and there is some evidence that adiponectin interferes with other aspects of cholesterol processing (Furukawa *et al.*, 2004; Matsuura *et al.*, 2007). This raises the possibility of cholesterol depletion as a mechanism for the adiponectin-mediated inhibition of ET-1 vasoconstriction. This

type of adiponectin action would be unlikely to fully block ET-1 action, as capacitative calcium entry may still occur, but this is also consistent with the observation of an approximate halving of the ET-1 response under the current conditions. However, such a proposal is highly speculative and further studies are needed to determine the mechanism via which adiponectin is able to modulate ET-1 action.

Several groups have proposed that the hemodynamic effects of insulin are regulated by the balance between the vasodilator and vasoconstrictor effects of NO and ET-1 respectively (Cardillo *et al.*, 1999b; Eringa *et al.*, 2002; Verma *et al.*, 2001). Thus, the ability of adiponectin to oppose ET-1-mediated vasoconstriction may be an important aspect of its insulin sensitising actions, leading to enhanced insulin action in muscle due to improved glucose and insulin supply to myocytes (Clark *et al.*, 2003). In addition, the actions of ET-1 and NO have been reported to be mutually antagonistic, allowing any imbalance, such as those seen in obesity or insulin resistance, to be amplified (Mather *et al.*, 2002). Mather *et al.* (2004) demonstrated that ET_A receptor blockade increased the amount of bioavailable NO in obese, but not diabetic or lean, individuals. They explained this as being due to a dysregulation of the feedback system between ET-1 and NO and proposed that the increased activity of ET-1 in obesity is due to some feature of obesity itself, as it was not seen in the other subject groups (Mather *et al.*, 2004). The hypoadiponectinemia present in obesity, and consequent reduction in the limiting effects of adiponectin on ET-1-mediated vasoconstriction, is a potential activator of this positive-feedback cycle. Progressive dysregulation of the relationship between ET-1 and NO may then contribute to the endothelial dysfunction observed in hypertension and type 2 diabetes (Mather *et al.*, 2004). Therefore, in the absence of adiponectin resistance, therapeutic targeting of adiponectin may redress the imbalance between ET-1 and NO in insulin resistance and hypertension, and improve disease outcomes in these states.

In summary, this study has identified a specific activity of adiponectin to oppose the vasoconstrictor effects of ET-1 and its associated metabolic effects. The lack of such an effect once vasoconstriction is established or against NE indicates that adiponectin is unlikely to be acting via a NO-dependent mechanism. These findings suggest that

an interaction between adiponectin and ET-1 may contribute to the maintenance of normal vascular tone. Imbalance in this relationship in obesity may contribute to the development of insulin resistance and cardiovascular disease.

CHAPTER 4 – MODULATION OF ADIPONECTIN AND ENDOTHELIN-1 ACTION IN INSULIN RESISTANCE

4.1 Introduction

The preceding work demonstrated a novel action of adiponectin in an intact vascular system. However, the actions of adiponectin may potentially be of more importance as insulin effects diminish. Intact adiponectin action in insulin resistance may provide a therapeutic target for improving insulin sensitivity. Conversely, impairments in adiponectin action may contribute to the pathological development of insulin resistance.

Numerous studies have reported adiponectin-mediated insulin sensitisation, including alleviation of insulin resistance and diabetes by adiponectin replenishment (Yamauchi *et al.*, 2001; Yamauchi *et al.*, 2003). Similarly, Satoh *et al.* (2005) demonstrated adiponectin-mediated improvements in insulin-stimulated glucose infusion rate and glucose uptake in insulin resistant rats. Therefore, therapeutic targeting of adiponectin activity may be beneficial, particularly if these pathways remain functional in insulin resistance.

Conversely, there have been reports that insulin resistant subjects also show resistance to the actions of adiponectin. Plasma adiponectin concentration increases with age, despite impaired insulin sensitivity and increasing hypertension, suggesting the adiponectin may be less effective (Iwashima *et al.*, 2004). Attenuation of adiponectin action on glucose homeostasis, fatty acid oxidation and insulin sensitivity has been demonstrated in obese subjects, and genetic and diet-induced animal models of insulin resistance (Bruce *et al.*, 2005; Lin *et al.*, 2007; Mullen *et al.*, 2007; Mullen *et al.*, 2009). This occurs independently of insulin resistance, as adiponectin resistance was observed in rats fed a high-unsaturated fat diet, which did not affect insulin sensitivity (Mullen *et al.*, 2007). In a later study, Mullen *et al.* (2009) found that resistance to the effects of globular adiponectin on fatty acid oxidation occurred within 3 days of

feeding rats a high-fat diet. The adiponectin resistance preceded the development of increased lipid accumulation after 2 weeks of the diet, and impairments in insulin-mediated glucose uptake, which were not recorded until 4 weeks of high-fat feeding (Mullen *et al.*, 2009). In addition, based on their findings in a genetic model of insulin resistance, Lin *et al.* (2007) suggested that deterioration in adiponectin activity may contribute to the progression from insulin resistance to diabetes. These data suggest that adiponectin resistance may be a primary event independent of insulin resistance.

Previous studies have investigated adiponectin action in high-fat fed animals. Yamauchi *et al.* (2001) showed that adiponectin is decreased following high-fat feeding in mice, and that chronic adiponectin administration improved insulin sensitivity and reduced the increase in adipose mass associated with high-fat feeding, despite a higher food intake. Meanwhile, adenoviral overexpression of adiponectin prevented high-fat feeding induced insulin resistance (Satoh *et al.*, 2005). However, these studies have not examined adiponectin action acutely in insulin resistance, nor the effects of adiponectin in the vasculature.

High-fat feeding may provide a better model of human insulin resistance than models based on genetic manipulation, as it more closely represents the events leading to insulin resistance in humans eating a westernised diet. Additionally, the high-fat fed rat is a model of insulin resistance early in the pathological progression, a stage which is thought to provide the best opportunity to improve insulin sensitivity and prevent the development of type 2 diabetes. Reports are mixed as to whether high-fat feeding increases body weight, possibly explained by variations between mice and rats and in the length of intervention, but consistently describe increased adiposity. High-fat feeding causes increased blood pressure, reduced endothelium-dependent vasodilation and insulin resistance, and these effects often occur in the absence of differences in food intake (Bourgoin *et al.*, 2008; Guang-Yao *et al.*, 2006; Henriksen *et al.*, 2008; Noronha *et al.*, 2005). In addition, high-fat fed rats exhibit impaired insulin-mediated microvascular perfusion (St-Pierre *et al.*, 2010).

The primary aim of this study was to determine whether the acute inhibitory action of adiponectin on ET-1-mediated vasoconstriction was retained in insulin resistant

animals. However, the vasoconstrictor response to ET-1 was found to be attenuated following high-fat feeding, leading to an examination of the interaction between ET-1 and inducible nitric oxide synthase (iNOS) in insulin resistance.

4.2 Materials and Methods

4.2.1 Animals

All experiments were approved by the University of Tasmania Animal Ethics Committee and were performed in accordance with the guidelines set out under the NHMRC Code of Practice for the care and use of animals for scientific purposes seventh edition (2004). Male Sprague-Dawley Rats (*n*=74) were housed at 21±1°C under a 12h light/dark cycle, and provided with food and water *ad libitum*. Rats were obtained at 3-4 weeks of age and fed a high-fat diet (19.4% protein, 36% lipid, 35.2% carbohydrate, 9.4% crude fibre by weight; Specialty Feeds, Glen Forrest, WA, Aus) for 4 weeks. An age-matched control group was fed normal chow (22% protein, 9% lipid, 61.4% carbohydrate and 7.6% crude fibre by weight; Barastoc Stockfeed, Pakenham, VIC, Aus). The enriched fat content of this high-fat diet, with energy contribution from fat increased from 19% to 60%, is compensated for largely by a reduction in calories provided as carbohydrate (Table 4.1).

Table 4.1: Relative energy contribution of macronutrients in the normal and high-fat diets.

	Control Diet	**High-Fat Diet**
	% Calories	% Calories
Protein	21	14
Total Fat	**19**	**60**
Carbohydrate	60	26

4.2.2 Perfused rat hindlimb

The constant flow, pump-perfused rat hindlimb procedure was conducted as described in Sections 3.2.2 and 3.2.3, with minor adjustments. Before removal of the testes, the epididymal fat pads were separated above the deferential artery and the combined wet weight was recorded. Immediately before the cannulas were inserted, 0.5mL of blood was collected intracardially and centrifuged to separate the erthrocytes from the plasma, which was stored at -20°C for later use in biochemical assays.

N-(3-(Aminomethyl)benzyl)acetamidine (1400W) is a potent and selective inhibitor of the inducible isoform of NOS (iNOS), inhibiting 99% of iNOS activity (Garvey *et al.*, 1997). Garvey *et al.* (1997) showed that 1400W is 5000-fold more potent against iNOS than either eNOS or nNOS *in vitro*, and greater than 1000-fold more potent against rat iNOS than eNOS in isolated rat aortic rings. Additionally, inhibition of eNOS or nNOS by 1400W is rapidly reversible, compared to effectively irreversible iNOS inhibition (Garvey *et al.*, 1997). In NOS inhibition experiments, 1400W or the general NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (both from Sigma, St Louis, MO, USA) was added to the buffer reservoir to a final concentration of 10μM. Thus, the hindlimb was exposed to the inhibitors throughout the equilibration period and for the duration of the experiment. ET-1 (Calbiochem, San Diego, CA, USA) was infused at 1/200th of the pump flow rate, providing final concentrations of 1nM or 3nM to the hindlimb. All other details are as described in Section 3.2.3. Individual protocols are shown in Figure 4.1.

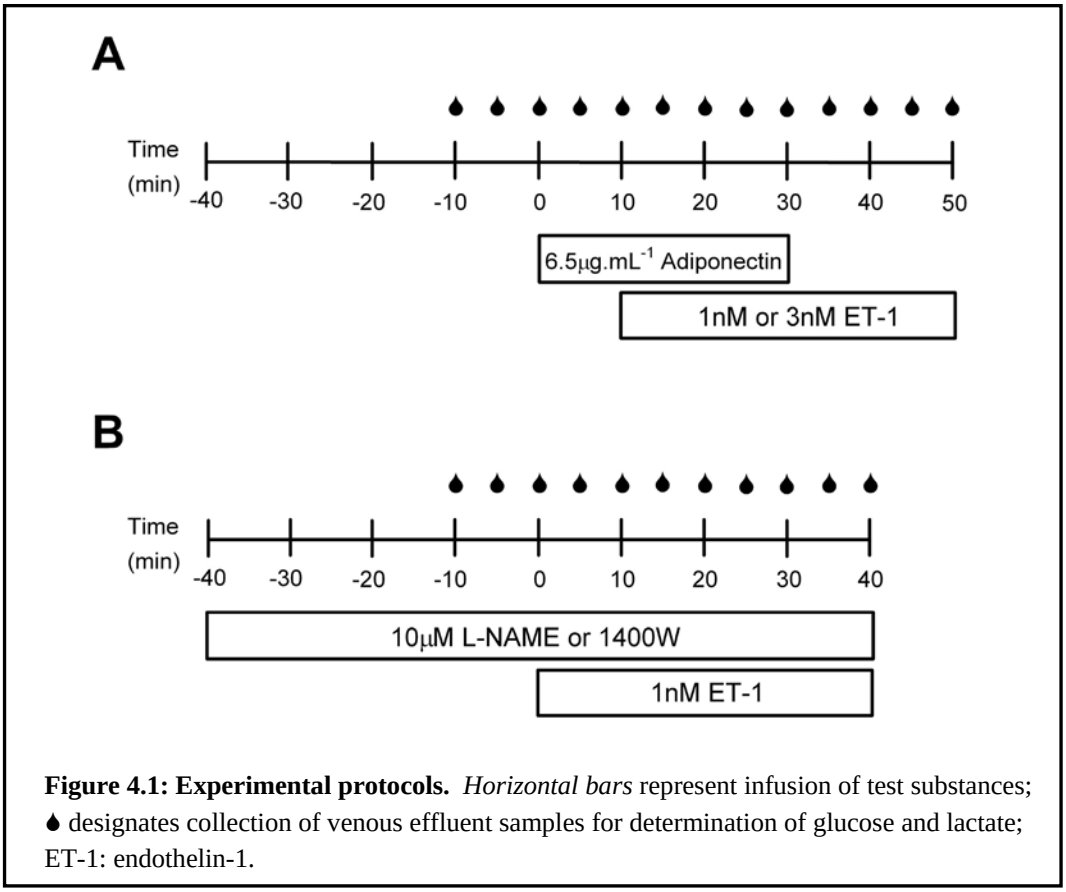

Figure 4.1: Experimental protocols. *Horizontal bars* represent infusion of test substances; ♦ designates collection of venous effluent samples for determination of glucose and lactate; ET-1: endothelin-1.

4.2.3 Biochemical Measurements

Glucose and lactate determinations were made using a glucose analyser (Model 2300 Stat Plus, Yellow Springs Instruments), and hindlimb uptake or release data respectively were derived from the product of flow, arterio-venous (A-V) concentration difference and perfused muscle mass. Plasma concentrations of insulin (Mercodia AB, Sweden), ET-1 (Biomedica, Austria) and adiponectin (Phoenix Pharmaceuticals, Burlingame, CA, USA) were determined by ELISA.

4.2.4 Statistics

SigmaStat (SPSS Science, Chicago, IL, USA) was used to conduct all statistical analyses. Single point data sets were compared via Students *t*-test or one way ANOVA as appropriate. Time course data were analysed using two-way repeated measures ANOVA. Differences between conditions were assessed using the Student-Neuman-Keuls post hoc test. Significance was assumed at the level of $p<0.05$. Data are presented as means ± standard error; if error bars are not visible, they are within the symbol.

4.3 Results

4.3.1 Four week high-fat feeding causes insulin resistance

4.3.1.1 High-fat feeding increases adiposity

Four week high-fat feeding resulted in a small but statistically significant increase in body weight (242±5g vs. 230±3g with the normal diet; $p<0.001$; Figure 4.2A). This was accompanied by a large increase in abdominal adiposity, as measured via wet weight of the epididymal fat pads (2.9±0.1g vs. 1.6±0.1g with the normal diet; $p<0.001$; Figure 4.2B). The relative increase in epididymal fat was much greater than the increase in body weight, and the significant increase persisted when epididymal fat weight was expressed as a percentage of total body weight (1.17±0.04% vs. 0.71±0.02% with the normal diet; $p<0.001$). Therefore, the 4 week high-fat fed rat is primarily a model of increased adiposity, and not obesity. This finding is in agreement with another recent study, although in that case no change in body weight was observed (St-Pierre *et al.*, 2010). The discrepancy between these two studies may be due to minor differences in the response between rat strains or the age at which high-fat feeding was initiated, as the rats may have commenced dietary intervention at a slightly earlier age in the present study. Although statistically significant, the increase in body weight was modest and so may not actually be biologically significant. Thus for most purposes these models are considered comparable.

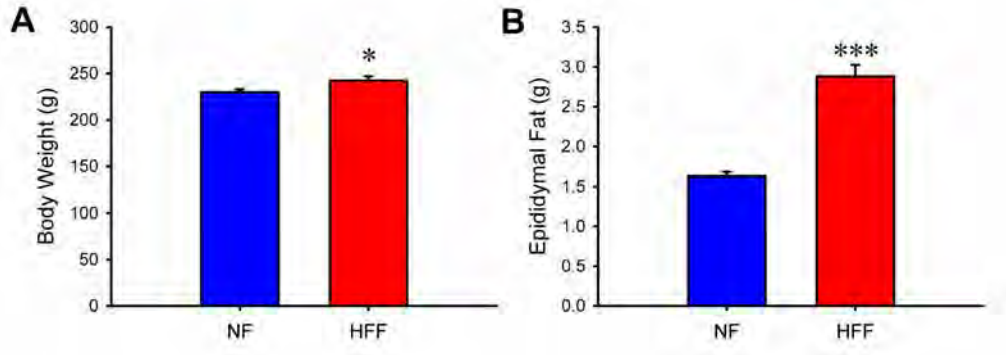

Figure 4.2: High-fat feeding increases body weight and adiposity. Changes in (A) body weight and (B) epididymal fat pad weight following 4 week high-fat feeding. NF: normal chow-fed rats (n=41); HFF: high-fat fed rats (n=46). * $p<0.05$, *** $p<0.001$, significantly different from normal diet.

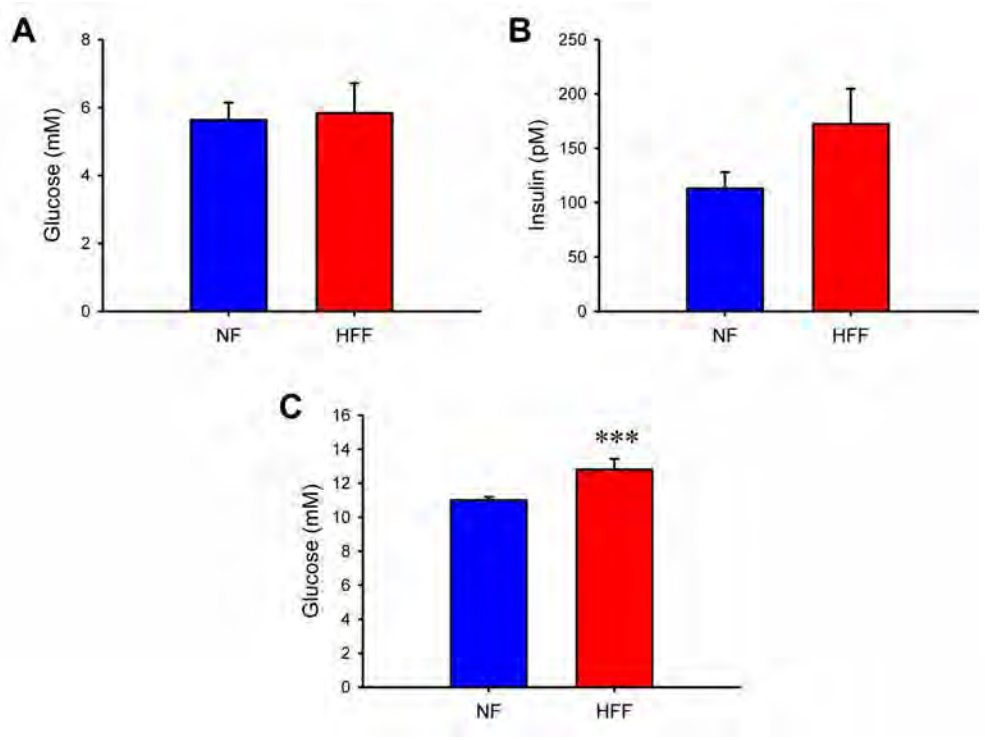

Figure 4.3: High-fat feeding induces insulin resistance. Fasting plasma concentrations of (A) glucose and (B) insulin, and (C) fed plasma glucose. NF: normal chow-fed rats; HFF: high-fat fed rats. *** $p<0.001$, significantly different from normal diet (n=4-12).

4.3.1.2 High-fat feeding reduces insulin sensitivity

Fasting plasma glucose was unchanged following 4 week high-fat feeding (Figure 4.3A). However, there was a trend for increased fasting plasma insulin concentrations in high-fat fed rats (172±33pM vs. 113±15pM with the normal diet; p=0.084; Figure 4.3B), suggesting increased insulin levels were required to maintain normoglycemia. Additionally, significant hyperglycemia was exposed in the fed state (Figure 4.3C), indicating a possible reduction in beta cell function. Together, these data suggest that 4 week high-fat feeding altered insulin sensitivity.

4.3.1.3 Plasma levels of ET-1 and adiponectin following high-fat feeding

High-fat feeding did not significantly alter plasma concentrations of adiponectin (6.8±0.5µg.mL^{-1} vs. 7.5±0.55µg.mL^{-1} with the normal diet; n=10; p=0.359; Figure 4.4A), or ET-1 (3.4±1.2pM vs. 2.0±0.5pM with the normal diet; n=4; p=0.323; Figure 4.4B). However, adiponectin and ET-1 levels were slightly reduced and increased respectively. Additionally, ET-1 concentrations may be locally elevated in vascular tissue of high-fat fed rats, which may not be reflected in plasma concentrations

(Bourgoin *et al.*, 2008). Alternatively, this model may represent a stage in the pathological process prior to changes in these plasma peptide concentrations occurring.

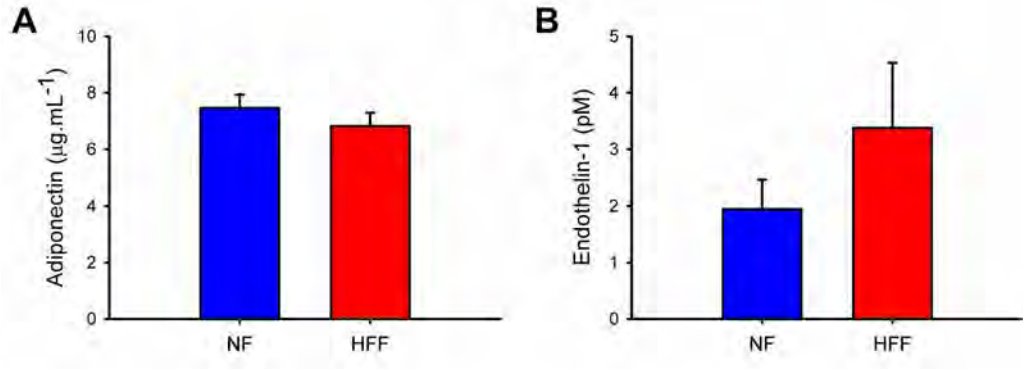

Figure 4.4: Adiponectin and Endothelin-1 levels in insulin resistance. (A) Plasma concentrations of adiponectin (*n*=10). (B) Plasma Endothelin-1 levels (*n*=4). NF: normal chow-fed rats; HFF: high-fat fed rats.

4.3.2 Adiponectin is ineffective against ET-1 in insulin resistance

Under basal conditions at a flow rate of 5mL.min^{-1}, average measurements in high-fat fed rats were perfusion pressure: 28.6±0.5mmHg, oxygen uptake: 10.3±0.3µmol.g^{-1}.h^{-1}, glucose uptake: 5.6±0.5µmol.g^{-1}.h^{-1}, and lactate release: 18.3±0.7µmol.g^{-1}.h^{-1} (n=47). These parameters were not significantly different from basal measurements in normal animals. As in the normal-chow fed animals shown in Chapter 3, adiponectin alone did not alter perfusion pressure in rats fed a high-fat diet (Figure 4.5A). Adiponectin-mediated glucose uptake was lower in high-fat fed animals than those fed normal chow, potentially indicating a loss of adiponectin action. However, the change in glucose uptake was not different between adiponectin and vehicle infused hindlimbs, suggesting that the increased glucose uptake with adiponectin alone was an artefact of the vehicle (Figure 4.5C).

Adiponectin had no effect on ET-1-induced vasoconstriction in high-fat fed rats, in contrast to the previous findings in normal animals. This result was consistent whether adiponectin was tested against the original 1nM ET-1 dose or at a similar perfusion pressure using 3nM ET-1 (Figure 4.5 A and B). Glucose uptake was higher in high-fat fed rat hindlimbs infused with adiponectin plus 1nM ET-1, compared to vehicle plus

1nM ET-1 (Figure 4.5C). This contrasts with the findings in normal animals shown in Chapter 3, where adiponectin co-treatment reduced ET-1-mediated glucose uptake.

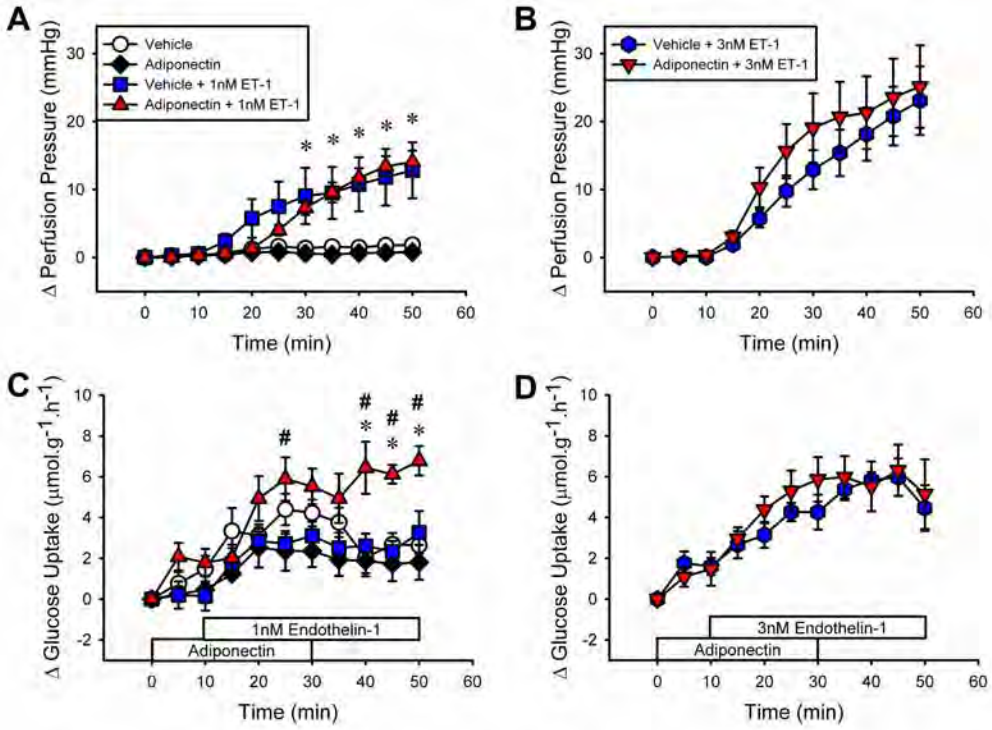

Figure 4.5: Effects of adiponectin in high-fat fed animals. Effect of adiponectin on basal and 1nM ET-1-mediated changes (left) or 3nM ET-1-mediated changes (right) in (A-B) perfusion pressure, and (C-D) glucose uptake. Substances were infused at the times indicated according to Figure 4.1A. Data are normalised to *t*=0min. * Significantly different from vehicle and # significantly different from vehicle + ET-1 ($p<0.05$; n=4-8).

It is possible that this indicates an unmasking of the effect of adiponectin alone in the presence of diminished ET-1 constriction following high-fat feeding. As it is proposed that the metabolic effects of ET-1 are entirely dependent on the vasoconstriction (Kolka *et al.*, 2005), greater dilation in the presence of a reduced ET-1 response may allow greater access for adiponectin. Indeed, the small effect of ET-1 may be enhancing adiponectin action by limiting flow to the non-nutritive route and thereby increasing exposure of myocytes to adiponectin. No difference in glucose uptake in response to 3nM ET-1 was observed between rats treated with adiponectin or vehicle. Adiponectin also had no effect on 3nM ET-1-mediated oxygen uptake ($1.5\pm0.3\mu mol.g^{-1}.h^{-1}$ vs. $1.1\pm0.4\mu mol.g^{-1}.h^{-1}$ with vehicle + ET-1; NS) or lactate release ($17.2\pm1.4\mu mol.g^{-1}.h^{-1}$ vs. $17.5\pm5.2\mu mol.g^{-1}.h^{-1}$ with vehicle + ET-1; NS) in high-fat fed rats.

4.3.3 The response to ET-1 is reduced following high-fat feeding

Perfusion pressure development in response to ET-1 was significantly reduced in high-fat fed animals compared to rats fed normal chow. The diminished ET-1 effect was apparent at both the 1nM and 3nM ET-1 doses (Figure 4.6A). These differences were not due to physical modifications or other basal variation between the animals, as no significant differences were observed prior to ET-1 infusion (Figure 4.6). The metabolic effects of ET-1 were similarly attenuated, with the effect of a given ET-1 dose lower in the high-fat than the normal-chow fed animals on each measure, although the differences were not significant in all cases (Figure 4.6). Glucose uptake induced by 1nM ET-1 was significantly reduced in high-fat fed rats, while the effect of 3nM ET-1 approached significance (4.6±1.1μmol.g^{-1}.h^{-1} vs. 8.7±0.9μmol.g^{-1}.h^{-1} with the normal diet at 50min; p=0.064).

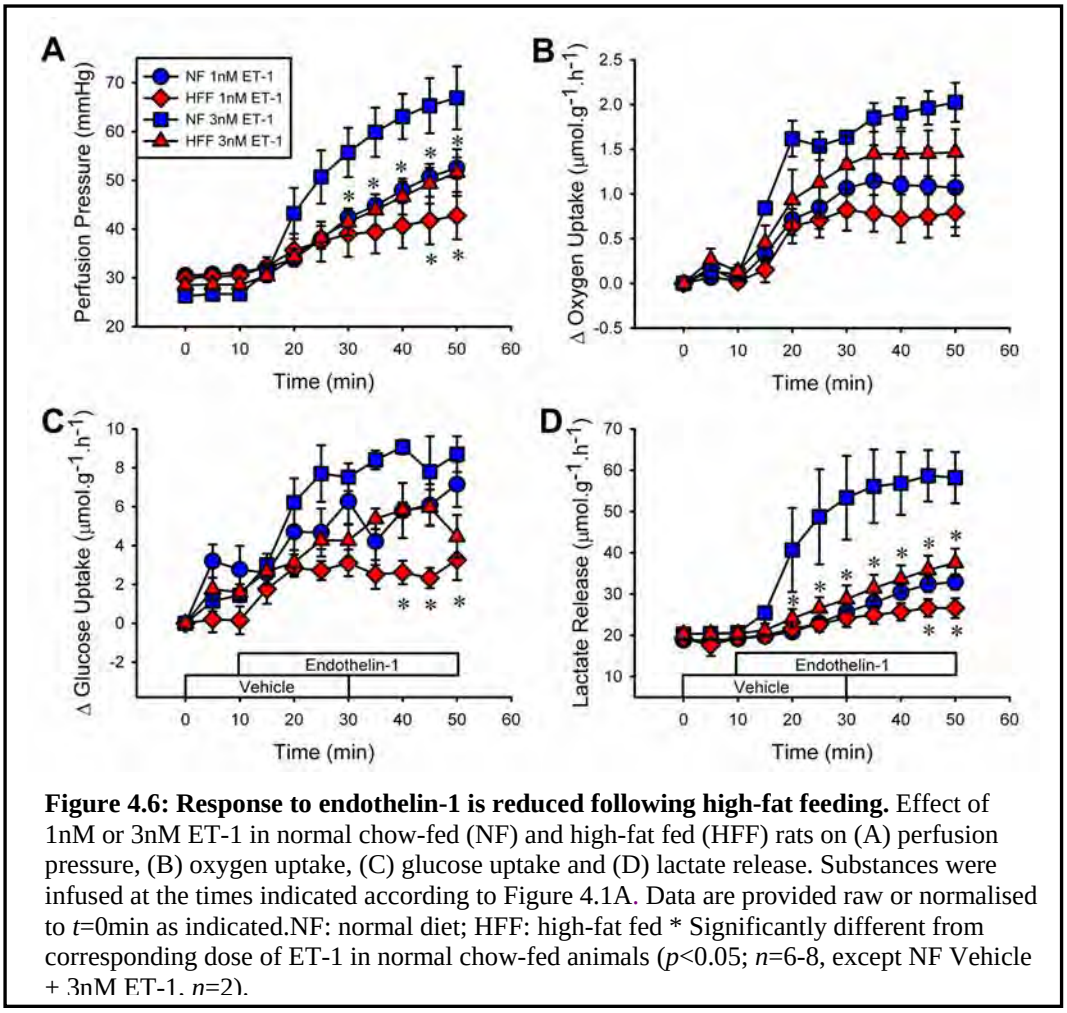

Figure 4.6: Response to endothelin-1 is reduced following high-fat feeding. Effect of 1nM or 3nM ET-1 in normal chow-fed (NF) and high-fat fed (HFF) rats on (A) perfusion pressure, (B) oxygen uptake, (C) glucose uptake and (D) lactate release. Substances were infused at the times indicated according to Figure 4.1A. Data are provided raw or normalised to t=0min as indicated.NF: normal diet; HFF: high-fat fed * Significantly different from corresponding dose of ET-1 in normal chow-fed animals (p<0.05; n=6-8, except NF Vehicle + 3nM ET-1, n=2).

4.3.4 Inhibition of nitric oxide synthase restores normal ET-1 response in high-fat fed rats

4.3.4.1 Nitric oxide synthase inhibition with L-NAME

Suppression of all NOS isoforms by L-NAME did not alter basal perfusion pressure, which is consistent with the perfused rat hindlimb being dilated basally. However, L-NAME treatment resulted in a significantly increased response to 1nM ET-1 in animals fed both diets (Figure 4.7B). These altered responses were accompanied by a significant increase in ET-1-stimulated oxygen uptake in normal animals and lactate release following high-fat feeding, but no other significant metabolic changes (Figure 4.7 D - F). ET-1-mediated perfusion pressure was significantly increased in the normal animals treated with L-NAME and ET-1, compared to ET-1 alone. This suggests that a small amount of NO, which partially restricts ET-1 vasoconstriction, is produced under normal conditions. The vasoconstrictor response to ET-1 in high-fat fed animals was restored in the presence of L-NAME, so that the perfusion pressure was no longer different to that of the normal-chow fed animals (Figure 4.7B). These experiments indicate that the discrepancy in the ET-1 response between normal-chow and high-fat fed animals is due to dysregulation of NO production.

4.3.4.2 Specific inhibition of inducible nitric oxide synthase

The finding of increased NOS activity in insulin resistance was contrary to numerous literature reports of reduced NO bioavailability (see Section 1.5). However, there have been reports that the inducible NOS (iNOS) isoform is upregulated in insulin resistance (Dallaire *et al.*, 2008; Fujimoto *et al*., 2005; Noronha *et al.*, 2005; Perrault & Marette, 2001). Therefore, iNOS was specifically targeted using the iNOS inhibitor 1400W, which is at least 1000-fold more selective for iNOS than either eNOS or nNOS (Garvey *et al.*, 1997). When this inhibitor was present before and during ET-1 infusion, ET-1-mediated perfusion pressure was significantly increased in the high-fat fed rats (Figure 4.7C). Once again, this was not due to basal variations in perfusion pressure between the dietary cohorts (Figure 4.7C). Meanwhile, there was no change in perfusion pressure in normal-chow fed animals. Therefore, specific iNOS inhibition restored the ET-1 vasoconstrictor response following high-fat feeding to a level that was not significantly different from the same treatment in normal-chow fed rats, or from the effect of ET-1 alone in normal animals. No significant metabolic effect of

iNOS inhibition was apparent in either dietary group (Figure 4.6 D-F). The significant effect of iNOS inhibition in high-fat fed, but not control animals, indicates that increased iNOS activity is responsible for the diminished ET-1 response in insulin resistance. Additionally, the difference in the effects of the two inhibitors on ET-1 action in normal-chow fed animals (perfusion pressure 66.0±5.9mmHg with L-NAME vs. 52.2±7.9mmHg with 1400W at 40min; p=0.045) indicates that the low level NO produced under normal conditions is most likely derived from eNOS or nNOS.

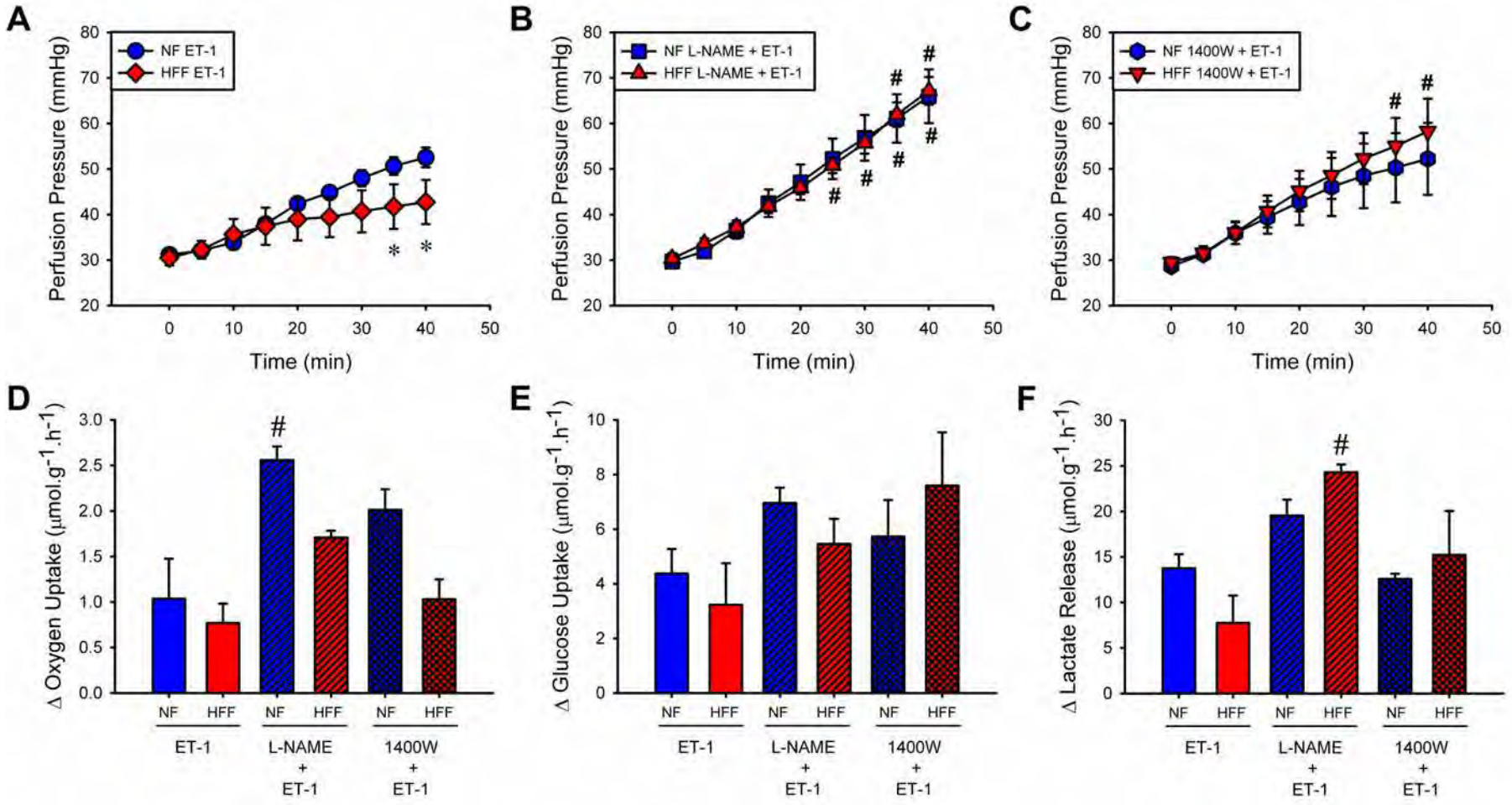

Figure 4.7: Inhibition of NOS restores endothelin-1 response in high-fat fed rats. (A-C) Comparative ET-1-induced perfusion pressure in normal or high-fat fed rats in the presence of (A) vehicle, (B) L-NAME, or (C) 1400W. (D-F) ET-1 response in normal and high-fat fed rats in the presence or absence of NOS inhibitors on (D) oxygen uptake, (E) glucose uptake, and (F) lactate release. Data are normalised to *t*=0min, according to Figure 4.1B. NF: normal diet; HFF: high0fat fed. * Significantly different from corresponding treatment in normal fed rats and # significantly different from ET-1 alone in corresponding diet ($p<0.05$; n=5-8).

4.4 Discussion

Adiponectin has been widely described as an insulin sensitiser, but whether these actions are maintained in the insulin resistant state remains unclear. A common animal model of insulin resistance, the high-fat fed rat, was used to address this question. Four weeks of high-fat feeding induced insulin resistance with increased adiposity, in the absence of overt obesity. In these animals, adiponectin was unable to oppose ET-1-mediated vasoconstriction, in the manner previously demonstrated in normal-chow fed rats. The lack of effect suggests that the vascular actions of adiponectin are restricted in insulin resistance, possibly implicating this loss of action in the development of insulin resistance. However, interpretation of this data is impeded by the finding that ET-1 vasoconstriction was also modulated by high-fat feeding. Using pharmacological inhibitors, I was able to attribute this variation to an increase in iNOS-derived NO in the model of insulin resistance.

Studies have consistently shown that endogenous ET-1 activity is increased in obesity, insulin resistance, diabetes and hypertension (Cardillo *et al.*, 2004; Miller *et al.*, 2002; Cardillo *et al.*, 2002; Elgebaly *et al.*, 2008; Cardillo *et al.*, 1999a). Similarly, a shift towards insulin-stimulated constriction, indicating increased ET-1 activity, has been described in rats fed a high-fat, high-sucrose diet (Bourgoin *et al.*, 2008). Enhanced ET-1 action is most likely due to the increased ET-1 production observed in these states (Campia *et al.*, 2004; Cardillo *et al.*, 2002; Miller *et al.*, 2002). The exception to these studies is a report of impaired vasodilation in response to ET_A receptor blockade in type 2 diabetics, although that may be accounted for by the normal plasma ET-1 levels in those subjects (McAuley *et al.*, 2000b). However, there are conflicting reports of both enhanced and impaired constrictor responses to exogenous ET-1 under conditions of metabolic stress. Increased responses to exogenous ET-1 have been described in hypertension in both rats and humans (Cardillo *et al.*, 1999a; Juan *et al.*, 1998) and in animal models of type 1 diabetes (Alabadí *et al.*, 2004; Lloréns *et al.*, 2004). ET-1-mediated constriction is also enhanced in genetic and fructose-fed rat models of insulin resistance (Katakam *et al.*, 2001; Romanko *et al.*, 2009; Hopfner *et al.*, 1998; Lesniewski *et al.*, 2008), although Simandle *et al.* (2005) found that contractile responses to ET-1 were unchanged following fructose-feeding. The

majority of authors have proposed that the augmented ET-1 action is attributable to either an increase in the number or activity of the ET_A receptor or a reduction in NO bioavailability, particularly in response to ET_B activation (Juan *et al.*, 1998; Alabadí *et al.*, 2004; Lloréns *et al.*, 2004; Katakam *et al.*, 2001). Conversely, a number of studies have demonstrated a decreased response to ET-1, in agreement with the present work. Reduced ET-1 constrictor responses were observed in hypertensive patients and rats (Ferro *et al.*, 2002; Amiri *et al.*, 2010). Similarly, ET-1 action was diminished in obese Zucker rats (Katakam *et al.*, 2006; Romanko & Stepp, 2005) and in type 2 diabetic subjects (Cardillo *et al.*, 2002; McAuley *et al.*, 2000a; Nugent *et al.*, 1996). It has been widely suggested that these changes are due to downregulation of the ET_A receptor or constrictor pathways (Cardillo *et al.*, 2002; Romanko & Stepp, 2005; Amiri *et al.*, 2010), in contrast to the previous studies suggesting upregulation. Such an adaptation may occur as a compensatory response to chronically elevated ET-1, and indeed Hirata *et al.* (1988) showed that 24h treatment of vascular smooth muscle cells with ET-1 decreased ET-1 binding capacity without affecting binding affinity, indicating a decrease in total ET receptor number. However, ET_A receptor expression was normal in obese Zucker rats (Katakam *et al.*, 2006) and unaffected by ET-1 overexpression (Hocher *et al.*, 2004), indicating this may not be occurring *in vivo*. Katakham *et al.* (2006) alternatively suggested that ET-1 receptor activation and calcium elevation are uncoupled in obese Zucker rats, as ET-1-stimulated increases in intracellular calcium were lower despite full receptor activation.

Variation in ET-1 constrictor responses may be due to differences in the precise pathologies being examined. It has been suggested that hyperinsulinemia may be responsible for altered ET-1 action (Cardillo *et al.*, 2002; Hopfner *et al.*, 1998). Insulin increases circulating ET-1 levels, ET-1 activity, ET_A receptor expression and, as a result, blood pressure (Frank *et al.*, 1993; Hopfner *et al.*, 1998; Juan *et al.*, 2004). However, Lteif *et al.* (2008) conducted differentially dosed hyperinsulinemic clamps in lean and obese subjects, in order to elicit the same insulin-mediated NO release in both groups. Under these conditions, there was no difference in insulin-mediated dilation in the presence of ET_A receptor inhibition, despite insulin concentrations being 3-fold higher in obese patients. Therefore, the authors concluded that augmented ET-1 responses in obesity are not due to elevated insulin levels (Lteif *et*

al., 2008). Additionally, the association of enhanced ET-1 activity with hypertension and type 1 diabetes where insulin is presumably low, while ET-1 effects are reduced in type 2 diabetes, suggests that hyperinsulinemia does not account for increased ET-1 constrictor responses. McAuley *et al.* (2000b) suggested that hyperglycemia may lead to ET_A downregulation in type 2 diabetes, but the discrepant ET-1 responses in type 1 and 2 diabetes, which both exhibit hyperglycemia, would similarly counter this argument. Cardillo *et al.* (2004) reported increased endogenous ET-1 activity in obese but not lean hypertensive subjects, suggesting an association with obesity and not hypertension. In other studies, elevated ET-1 responses were observed in animal models of both insulin resistance and type 2 diabetes in the absence of obesity, indicating that obesity is not the causal factor (Romanko *et al.*, 2009; Elgebaly *et al.*, 2008). In a telling investigation by Lesniewski *et al.* (2008), an amplified ET-1 response was demonstrated in pre- and short-term type 2 diabetes in the Zucker diabetic fat rat. The enhanced ET-1 activity preceded the development of overt diabetes and obesity, and was lost in long-term diabetes (Lesniewski *et al.*, 2008). This data complements that of Romanko & Stepp (2005), where reduced vasoconstriction was observed in obese Zucker rats but not in the fructose-fed insulin resistant model. Based on all of the data discussed, it appears that ET-1 reactivity is increased is type 1 diabetes, hypertension and in the early stages of insulin resistance, possibly due to increased ET-1 concentrations and ET_A receptor activity. However, more severe insulin resistance and type 2 diabetes, particularly in the presence of obesity, leads to a diminution of ET-1 reactivity. This theory is consistent with the data of the current study, whereby ET-1 vasoconstrictor responses were impaired in high-fat fed rats; a model of insulin resistance with increased adiposity. Given that this was attributed to increased iNOS activity, it is proposed that the reduced ET-1 response is primarily associated with the inflammation present in these states.

I have identified increased iNOS activity as the cause of the reduced ET-1 response in the high-fat fed rat. Noronha *et al.* (2005) showed a very similar effect, with iNOS inhibition eliciting an increase in the constrictor response to phenylephrine in high-fat fed, but not lean, mice. Diminished ET-1-mediated constriction in Zucker obese rats has also been related to elevated basal NO, as the response was normalised in the presence of L-NAME (Katakam *et al.*, 2006). Furthermore, augmented iNOS activity

resulting in decreased vasoconstrictor responses has been associated with the development of hypotension (reviewed in Nathan, 1997). Although increased iNOS expression was not established in the current study, a number of previous investigations have demonstrated enrichment of iNOS in conditions of metabolic stress. iNOS mRNA was increased in aortas of high-fat fed mice, without a change in eNOS or nNOS expression (Noronha *et al.*, 2005). Elevated iNOS has also been described in liver, skeletal muscle and adipose tissue of genetic and diet-induced models of obesity and insulin resistance (Dallaire *et al.*, 2008; Fujimoto *et al.*, 2005; Perreault & Marette, 2001). High-fat fed mice had increased iNOS expression particularly in muscle, with some increase also in adipose tissue, but there was no rise in liver iNOS mRNA following high-fat feeding (Perreault & Marette, 2001). This is in contrast to the effects of an acute intralipid infusion, which predominantly augmented iNOS in liver (Charbonneau & Marette, 2010). A number of studies have shown that iNOS is induced by pro-inflammatory cytokines, particularly in combination (Kapur *et al.*, 1999; Bédard *et al.*, 1997). Therefore, it has been suggested that increased pro-inflammatory cytokines in adiposity, possibly derived from adipocytes within skeletal muscle, may trigger iNOS expression (Perreault & Marette, 2001). Additionally, transgenic overexpression of ET-1 caused increased iNOS expression, and this was also linked to inflammation (Hocher *et al.*, 2004). This suggests an alternate mechanism of iNOS upregulation, whereby elevated ET-1 levels may promote inflammation, leading to iNOS induction.

Activation of iNOS under normal conditions has a range of beneficial functions, most notably in immune responses to infection and inflammation (reviewed in Nathan, 1997). However, iNOS also affects a range of other processes, and so can be detrimental in excess. Enhanced iNOS activity impairs vascular function, reducing endothelium-dependent vasorelaxation (Kessler *et al.*, 1997; Gunnett *et al.*, 2001) and causing apoptosis of vascular smooth muscle cells (Iwashina *et al.*, 1998). Excess NO has been reported to decrease insulin-mediated glucose uptake in isolated muscle, indicating it also has direct effects on muscle metabolism (Kapur *et al.*, 1997). Upregulation of iNOS is associated with diminished insulin sensitivity, depressed hepatic insulin signalling, enhancement of the hepatic gluconeogenesis pathway and nitration of tyrosine residues (Bédard *et al.*, 1997; Charbonneau & Marette, 2010;

Fujimoto *et al.*, 2005). Inhibition of iNOS improved insulin sensitivity and signalling in ob/ob mice (Fujimoto *et al.*, 2005); while rosiglitazone improved insulin sensitivity in high-fat fed mice in conjunction with completely normalised iNOS levels (Dallaire *et al.*, 2008). Disruption of the iNOS gene protects mice from insulin resistance induced by both high-fat feeding and acute lipid infusion, without affecting insulin sensitivity and glucose homeostasis in normal animals (Perreault & Marette, 2001; Noronha *et al.*, 2005; Charbonneau & Marette, 2010). Thus, increased iNOS activity is likely to contribute to the pathology of insulin resistance.

Although excess ET-1 also has a range of detrimental effects, it too is beneficial when tightly regulated. ET-1 is increased in internal organs and non-working muscles during exercise, reducing blood flow to these areas, and ET_A receptor inhibition reduces flow in working muscle while preventing normal flow decreases in other tissues (Maeda *et al.*, 1997; Maeda *et al.*, 2002b). Thus, ET-1 plays an important role in redistributing blood flow to high demand areas during exercise. Additionally, ET-1 is produced in response to insulin (Yang & Li, 2008; Ferri *et al.*, 1995; Ferri *et al.*, 1996) and its localised effects are thought to improve insulin action, by redirecting blood flow away from the non-nutritive route (Kolka *et al.*, 2005). Thus, insulin stimulates both NO and ET-1 production, and it has been shown that it is the balance between the two that is important for insulin's hemodynamic action (Eringa *et al.*, 2002; Verma *et al.*, 2001). Meanwhile, iNOS produces markedly higher levels of NO than the constitutive NOS isoforms, and iNOS-induced dilation is likely to indiscriminately increase both nutritive and non-nutritive blood flow. Therefore, iNOS upregulation would be expected to interfere with the highly coordinated hemodynamic regulation, reducing muscle insulin sensitivity. This further implies that the benefits of vasodilation must be considered in context, and general dilators may not be particularly useful for the treatment of insulin resistance. Elevated activation of both ET-1 and iNOS in metabolic stress would maintain the balance between ET-1 and NO, but at a fundamentally increased level. This is supported by findings that endogenous ET-1 activity was not augmented in rats chronically fed a high-fat diet for 12 months (da Silva *et al.*, 2004), nor in patients with essential hypertension, despite an impaired response to exogenous ET-1 (Ferro *et al.*, 2002). Additionally, Hocher *et al.* (2004) demonstrated that ET-1 overexpression upregulates iNOS, causing blood

pressure to be unchanged. In the presence of increases in both vasoconstrictor and vasodilator signals, a relatively small stimulation of NO production through eNOS is unlikely to have any great additional effect. Thus, an increased state of balance between ET-1 and NO may contribute to the development of endothelial dysfunction and the loss of insulin's vascular actions.

The present work tends to suggest that insulin resistance is accompanied by resistance to adiponectin action. Specifically, the ability of adiponectin to acutely oppose ET-1 action was absent in high-fat fed rats. However, these results are inconclusive, as identical dose and pressure conditions of ET-1 in both dietary cohorts, against which to test adiponectin, could not be achieved. In addition, adiponectin inhibits iNOS induction and the concomitant NO production (Tao *et al.*, 2007). Activating AMPK, a kinase known to be involved in adiponectin signalling, reduces iNOS activity and protein expression, resulting in improved insulin sensitivity (Pilon *et al.*, 2004). Rosiglitazone-mediated normalisation of iNOS levels and improvements in insulin sensitivity in high-fat fed mice have been associated with markedly increased adiponectin concentrations, which are a hallmark of the PPARγ agonist insulin sensitising drugs (Dallaire *et al.*, 2008). These data suggest that adiponectin may have an inhibitory effect on iNOS activity. Therefore, the absence of adiponectin-mediated changes in perfusion pressure in high-fat fed rats demonstrated here may be complicated by conflicting effects of adiponectin to inhibit both ET-1 and iNOS. However, the above studies largely described chronic changes in iNOS gene expression, so an interaction between adiponectin and iNOS may not affect the acute adiponectin treatment of the current study. Furthermore, as discussed above, the vasodilatory effects of adiponectin are likely to be relatively modest, and thus any action is likely to be inconsequential in the presence of increased levels of both ET-1 and NO. Therefore, the primary aim of this study, to determine whether the inhibitory action of adiponectin on ET-1-mediated vasoconstriction was retained in insulin resistant animals, was unable to be properly addressed due to the modulation of the ET-1 response in insulin resistance.

This work has identified an attenuation of ET-1 action in insulin resistance, which is attributable to increased iNOS activity. Increases in both vasodilator and

vasoconstrictor stimuli may reduce responsiveness to NO-dependent vasodilators, and thus contribute to endothelial dysfunction and vascular defects associated with the metabolic syndrome.

CHAPTER 5 – THE HEMODYNAMIC AND METABOLIC ACTIONS OF ADIPONECTIN *IN VIVO*.

5.1 Introduction

Having examined the direct effects of adiponectin on skeletal muscle vasculature in the perfused rat hindlimb, the next aim was to investigate acute adiponectin action *in vivo*. As the perfused rat hindlimb includes mainly muscle, potential contributions to the metabolic and vascular actions of adiponectin from the liver and central nervous system are not able to be assessed using that technique. Non-recirculating perfusion of the hindlimb also washes out any endogenous circulating factors that may contribute to vascular tone, including adiponectin. Thus, perfused hindlimb experiments can not indicate whether exogenous adiponectin has any added effect above endogenous concentrations, a question particularly important when considering therapeutically increasing adiponectin levels. Furthermore, the perfused rat hindlimb does not allow for examination of microvascular redistribution under physiological conditions, due to the highly dilated basal state. In addition, the constricted perfused rat hindlimb technique used in previous investigations of this thesis was not particularly suitable for the evaluation of adiponectin-mediated insulin sensitisation. As insulin was able to completely abolish ET-1-mediated vasoconstriction (Kolka *et al.*, 2005), submaximal insulin doses would be required to observe an adiponectin effect, preventing the question of additivity or synergism from being addressed. Thus, whilst the perfused rat hindlimb provides a valuable system for the examination of vascular changes specifically in muscle, whole animal studies provide a more physiologically relevant system to investigate hemodynamic and metabolic influences.

The hyperinsulinemic euglycemic clamp is the current gold standard measure of insulin sensitivity. Insulin is infused systemically, stimulating glucose disposal and inhibiting hepatic glucose output, leading to reduced blood glucose. However, changes in glucose levels independent of insulin have biological effects including sympathetic nervous system activation (Donovan *et al.*, 1994), as well as affecting NO

release and insulin-stimulated vasodilation (Taubert *et al.*, 2004). To avoid these confounding effects glucose is continually infused in concert with the insulin, and the rate at which glucose infusion is required to maintain stable glycemia is a powerful indicator of whole body insulin sensitivity. This technique has been used widely for the examination of insulin sensitivity in conjunction with measures of both total blood flow and microvascular perfusion, in anaesthetised rats (Zhang *et al.*, 2004; Wheatley *et al.*, 2004; St-Pierre *et al.*, 2010; and reviewed in Clark *et al.*, 2003). Although anaesthetics have a range of biological effects, studies in conscious animals do not readily lend themselves to measures of blood flow, especially microvascular flow, which are subject to movement artefacts. Given that the hemodynamic actions of adiponectin were the primary focus of the current study, accurate blood flow measurements were crucial. Additionally, insulin has comparable effects on microvascular perfusion in anaesthetised animals and conscious human subjects (reviewed in Clark, 2008), indicating that the anaesthetic should not substantially affect the results of the current experiments.

Previous investigations of adiponectin action *in vivo* have demonstrated increased insulin sensitivity, in particular suppression of endogenous glucose production.

These studies have predominantly been conducted using chronic elevations in adiponectin, via either adenoviral or transgenic overexpression (Combs *et al.*, 2004; Kim *et al.*, 2007; Satoh *et al.*, 2005; Yamauchi *et al.*, 2003). Similarly, continuous infusion of adiponectin reduced body weight and restored insulin sensitivity in high-fat fed animals over a two week treatment period (Freubis *et al.*, 2001; Yamauchi *et al.*, 2001). In one study concerning the vasculature, angiogenesis was increased in rabbit corneas 14 days after adiponectin injection (Ouchi *et al.*, 2004). A few studies have also reported acute effects of adiponectin *in vivo*. Yamauchi *et al.* (2002) reported phosphorylation of AMPK and ACC in isolated myocytes within five minutes of adiponectin treatment. Globular adiponectin reduced the postprandial increase in free fatty acids within one hour of treatment and improved *in vivo* glucose homeostasis within two hours (Fruebis *et al.*, 2001); while a single injection of adiponectin was sufficient to abolish hyperglycemia in diabetic mice (Berg *et al.*, 2001). Meanwhile, acute infusion of adiponectin in the presence of physiological hyperinsulinemia improved insulin sensitivity via a significant suppression of hepatic

glucose production (Combs *et al.*, 2001). However, the acute effects of adiponectin on *in vivo* vasoactivity have not been described.

The aim of the current experiments was to determine if adiponectin acutely affects hemodynamics or metabolism *in vivo*. Given that the previously described vascular action of adiponectin in the perfused rat hindlimb was relatively modest and specific, it was of interest to see if it translates into a significant effect in the setting of complex hemodynamic regulatory mechanisms *in vivo*. The response to adiponectin was investigated, both alone and in the presence of a hyperinsulinemic clamp, to assess adiponectin-mediated insulin sensitisation. Additionally, the anaesthetised rat largely avoids the issue of altered ET-1 vasoconstriction in insulin resistance associated with the pre-constricted hindlimb, thus providing an alternate platform to examine the actions of adiponectin in insulin resistance.

5.2 Methods

5.2.1 Animals

All experiments were approved by the University of Tasmania Animal Ethics Committee and were performed in accordance with the guidelines set out under the NHMRC Code of Practice for the care and use of animals for scientific purposes seventh edition (2004). The majority of experiments were conducted on Hooded Wistar Rats (*n*=71), with Sprague-Dawley rats used for the low-dose adiponectin experiments when Hooded Wistars were no longer available. Male rats were housed at 21±1°C under a 12h light/dark cycle. Rats were provided with rat chow (Barastoc Stockfeed, Pakenham, VIC, Aus), containing 21.4% protein, 4.6% lipid, 68% carbohydrate and 6% crude fibre by weight, with added vitamins and minerals, and water *ad libitum*.

Rats for high-fat feeding studies were obtained at approximately 4 weeks of age and fed a high-fat diet (19.4% protein, 36% lipid, 35.2% carbohydrate, 9.4% crude fibre by weight; Specialty Feeds, Glen Forrest, WA, Aus) for 4 weeks. This protocol was designed so that the rats were age-matched to the control group at the time of experiment. The enriched fat content of this high-fat diet, with energy contribution from fat increased from 10% to 60%, is compensated for largely by a reduction in calories provided as carbohydrate (Table 5.1).

Table 5.1: Relative energy contribution of macronutrients in the normal and high-fat diets.

	Control Diet % Calories	**High-Fat Diet** % Calories
Protein	19	14
Total Fat	**10**	**60**
Carbohydrate	71	26

5.2.2 In vivo experiments

5.2.2.1 Surgery

Surgery was conducted on male rats weighing 240.5±2.3g (*n*=92) following an overnight fast. Animals were anaesthetised with an intraperitoneal injection of pentobarbital sodium (50mg.kg^{-1} body weight; Ilium Veterinary Laboratories, Smithfield, NSW, Australia). The rats were positioned on a heat pad at 37°C under a heat lamp to maintain body temperature. A ventral incision was made in the neck, and a tracheotomy was performed to allow the rat to freely breathe room air throughout the procedure. Both jugular veins were cannulated using polyethylene cannulas (PE-60, Intramedic®), which was secured in place with silk ligatures, to allow continuous infusion of anaesthetic and test substances. The carotid artery on one side was cannulated in the same way for arterial blood sampling. The arterial cannula was connected to a pressure transducer (Transpac IV, Abbott Critical Systems, Morgan Hill, CA, USA) to provide continuous blood pressure and heart rate measurements. A small area of skin (approximately 1cm^2) was removed from the medial side of each rear leg, to expose the femoral vessels. The superficial epigastric vessels of each leg were ligated to restrict blood flow to the femoral artery and allow accurate assessment of total limb blood flow via this vessel. The femoral artery of one leg was separated from the saphenous nerve and femoral vein, and an ultrasonic flow probe (Transonic™ Systems, VB series 0.5mm, Ithaca, NY, USA) was positioned around the artery between the junction of the rectus muscle and the epigastric artery branch point. The leg cavity was filled with lubricating gel (H-R, Mohawk Medical Supply, Utica, NY, USA) to provide good sound conductance. The flow probe was then attached to a flow meter (Model T106 ultrasonic volume flow meter, Transonic™ Systems, Ithaca, NY, USA).

5.2.2.2 Experimental procedures

Rats were maintained under anaesthesia using a variable rate, continuous infusion of pentobarbital sodium into the jugular vein. An equilibration period of 60min was allowed after completion of the surgery, to ensure stable anaesthesia. Following this, an arterial sample was collected for the determination of fasting blood and plasma

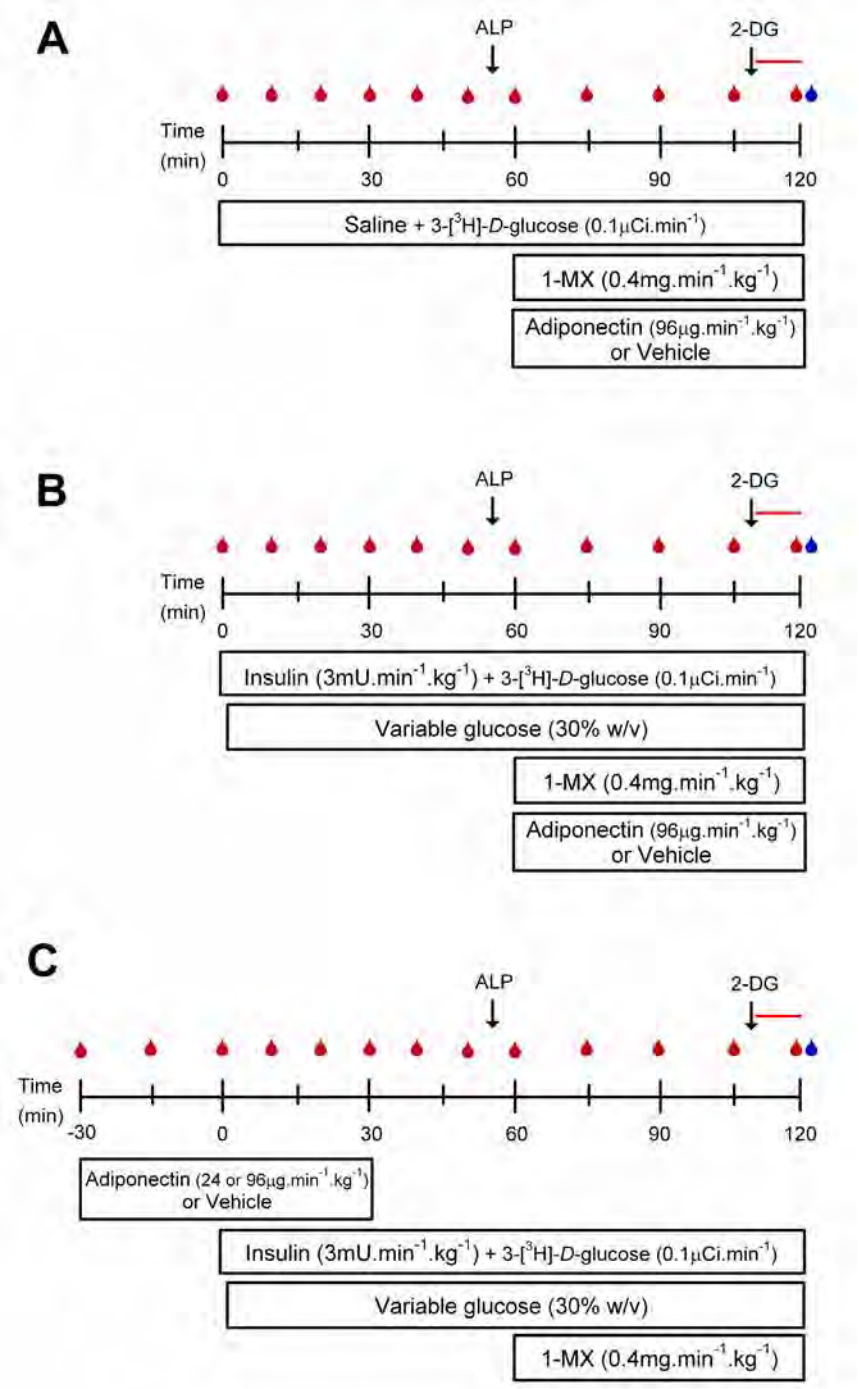

Figure 5.1: Experimental protocols. The *in vivo* actions of adiponectin or vehicle were examined alone (A) or during a hyperinsulinemic euglycemic clamp (B). The effects of pre-treatment and co-infusion of adiponectin or vehicle with insulin was also examined (C). 3-[^{3}H]-D-glucose was infused throughout all experiments to determine glucose turnover. An i.v. bolus of 2-deoxy-D-[1-^{14}C]-glucose (2-DG) was administered at 108min for the determination of muscle glucose uptake and arterial blood was continuously sampled over the last 10min of the protocol to provide a measure of circulating radioactivity. 1-MX was continuously infused for the last hour of experiments for the determination of microvascular perfusion. The muscles of the calf were excised and freeze-clamped in liquid nitrogen at the conclusion of the experiment.

Horizontal bars represent venous infusions, 🩸 designates collection of arterial blood samples for determination of blood glucose and lactate, 🩸 represents venous blood sample to determine of arterio-venous differences, — denotes continuous sampling of arterial blood to determine circulating 2-DG levels, and *arrows* indicate bolus injections of allopurinol (ALP) or radioactive 2-DG as indicated.

glucose. The effect of a 1hr adiponectin infusion (24 or 96μg.min^{-1}.kg^{-1}), alone or in the presence of insulin, was examined using the protocols shown in Figure 5.1. The effect of adiponectin was compared to the effect of the vehicle preparation extracted from non-transfected bacteria. The hyperinsulinemic euglycemic clamp technique was used to examine the effects of adiponectin on insulin action, in which insulin (Humulin R, Eli Lilly®, USA) was infused at a rate of 3mU.min^{-1}.kg^{-1} for 2hr. Insulin was diluted in heparinised saline containing 0.4% w/v bovine serum albumin. Physiological hyperinsulinemia was selected for these experiments to allow detection of adiponectin-mediated improvements in insulin sensitivity. During insulin infusion a variable rate infusion of 30% w/v glucose was used to maintain euglycemia. Blood glucose was measured every 10min during the first hour of the insulin infusion, and every 15min in the second hour when the effects of insulin reach a steady state. The rate of glucose infusion was used as a measure of whole body insulin sensitivity.

All infused substances were diluted in heparinised saline. Throughout the experiment, pressure, flow and heart rate data were continuously recorded using WINDAQ data acquisition software (DATAQ instruments, Akron, OH, USA). At the conclusion of the experimental protocol, the epididymal fat pads were excised and the wet weight recorded. Arterial and venous plasma samples collected throughout the experiment were stored at -20°C.

5.2.2.3 1-methylxanthine metabolism

Microvascular perfusion was assessed via a previously established method using the metabolism of exogenous 1-methylxanthine (1-MX; Sigma Aldrich, St. Louis, MO, USA) by the enzyme xanthine oxidase (Rattigan *et al.*, 1997b). Xanthine oxidase is located in the endothelium of capillaries and small arterioles in skeletal muscle (Jarasch *et al.*, 1986). As the number of perfused capillaries increases, so does the endothelial surface area exposed to the circulating 1-MX. Therefore the rate of 1-MX metabolism reflects changes in capillary perfusion.

1-MX is rapidly metabolised by xanthine oxidase, thus allopurinol, a specific inhibitor of xanthine oxidase, is used to partially inhibit its activity, particularly in non-muscle

tissue (Asp *et al.*, 1997; Emmerson *et al.*, 1987). Allopurinol (10μmol.kg^{-1}; Sigma Aldrich, St. Louis, MO, USA) was administered as a bolus via the carotid artery 5min prior to commencement of 1-MX infusion (0.1mg.min^{-1}.kg^{-1}), maintaining a constant arterial concentration of 1-MX throughout the experiment. 1-MX and allopurinol were both dissolved in 0.03% v/v 1M NaOH in heparinised saline and stored at -20°C.

Arterial and venous blood samples were collected in chilled tubes rapidly upon completion of the protocol. Plasma was obtained by immediate centrifugation, and 20μL was mixed with 80μL of 2M perchloric acid to deproteinate the sample. Concentrations of 1-MX were determined by reverse-phase high performance liquid chromatography as previously described (Rattigan *et al.*, 1997a). 1-MX metabolism was calculated by multiplying the arterial-venous 1-MX difference by femoral blood flow, and corrected for the volume accessible to 1-MX (0.871), which was determined from plasma concentrations after addition of 1-MX to whole rat blood.

5.2.2.4 Muscle glucose uptake

To assess the uptake of glucose into skeletal muscle, a 100μL bolus of 2-deoxy-*D*-[1-^{14}C]-glucose (2-DG; specific activity 56.0mCi.mmol^{-1}, Amersham Pharmacia Biotech, IL, USA) was administered via the jugular vein, 12min prior to completion of the experiment. The circulating concentration of radioactive glucose was determined from a sample of arterial blood constantly withdrawn at a rate of 50μL.min^{-1} for the final 10min of the protocol. Samples were centrifuged and radioactivity was assessed from 25μL of plasma.

At the conclusion of the experiment, whole calf muscle samples, consisting of the soleus, plantaris and gastrocnemius muscles were excised, freeze-clamped in liquid nitrogen and stored at -80°C. Frozen muscle samples were powdered under liquid nitrogen and 100mg of tissue was homogenised with 1mL water using a Heidolph™ silent crusher M (27000rpm.min^{-1}). Free and phosphorylated [^{14}C] 2-DG were separated by ion exchange chromatography using an anion exchange resin (AG® 1-X8, Bio-Rad Laboratories, CA).

Samples were combined with Biodegradable Counting Scintillant (Amersham Pharmacia Biotech, IL) and [^{14}C] radioactivity was determined using a scintillation counter (Tri-Carb 2800TR, Perkin Elmer Inc., IL, USA). Muscle 2-DG uptake (R'g), which reflects glucose uptake into the muscle, was calculated using the counts from the muscle and arterial samples, as previously described by others (Kraegen *et al.*, 1985; James *et al.*, 1985).

5.2.2.5 Rate of glucose turnover

The rate of glucose appearance and disappearance, or glucose turnover, was determined using a continuous infusion of 3-[^{3}H]-*D*-glucose (0.1μCi.min^{-1}; specific activity 16.6Ci.mmol^{-1}; Amersham Pharmacia Biotech, IL, USA). This was administered over the 2hr of the insulin, or corresponding saline, infusion. Arterial plasma samples were collected 15min prior to, and on completion of the experiment, and deproteinised using 2M perchloric acid. Samples were evaporated to dryness, in order to remove $^{3}H_2O$, and resuspended in distilled water. Biodegradable Counting Scintillant (Amersham Pharmacia Biotech, IL) was added to each sample and [^{3}H] radioactivity was determined using a scintillation counter (Tri-Carb 2800TR, Perkin Elmer Inc., IL, USA). The rates of appearance (Ra) and disappearance (Rd) of glucose calculated using the isotope dilution equation:

$$Ra = Rd = F / SA$$

* Where: F = the rate of tracer infusion

SA = the specific activity of glucose (calculated by dividing the plasma radioactivity by the glucose concentration).

5.2.3 Biochemical measurements

Glucose and lactate determinations were made using a glucose analyser (Model 2300 Stat Plus, Yellow Springs Instruments). Plasma concentrations of insulin (Mercodia AB, Sweden), ET-1 (Biomedica, Austria) and adiponectin (Phoenix Pharmaceuticals, Burlingame, CA, USA) were determined by ELISA.

5.2.4 Data analysis

Mean arterial blood pressure (MAP), mean heart rate (HR) and mean femoral blood flow (FBF) were calculated from 5sec sub-samples of data, representing approximately 300 measurements for each data point. Vascular resistance (VR) in the hindleg was calculated by dividing mean arterial pressure in mmHg by femoral blood flow in $mL.min^{-1}$ for each time point. Hindleg glucose uptake (HGU) was derived from the product of the arterio-venous (A-V) concentration difference and plasma flow.

5.2.5 Statistical analysis

SigmaStat (SPSS Science, Chicago, IL, USA) was used to conduct all statistical analyses. Single point data sets were compared via Students *t*-test or one way ANOVA as appropriate. Time course data were analysed using two-way repeated measures ANOVA. Differences between conditions were assessed using the Student-Neuman-Keuls post hoc test. Significance was assumed at the level of $p<0.05$. Data are presented as means ± standard error; if error bars are not visible, they are within the symbol.

5.3 Results

5.3.1 Effect of high-dose adiponectin on in vivo hemodynamics and glucose homeostasis

Preliminary experiments were conducted using adiponectin dosing based on the murine study by Combs *et al.* (2001), using a constant adiponectin infusion of 20μg.min^{-1}.kg^{-1} for 10min, reduced to 2μg.min^{-1}.kg^{-1} for the remainder of the 90min experiment. However, this dose of adiponectin, in the presence or absence of insulin, did not affect any measure of *in vivo* hemodynamics or glucose homeostasis (data not shown). Therefore, experiments were conducted using a high rate of adiponectin infusion, at 96μg.min^{-1}.kg^{-1}. These experiments were designed to produce supra-physiological circulating adiponectin concentrations, to determine whether adiponectin has any vascular activity at what were likely to be saturating levels.

5.3.1.1 Infusion of recombinant adiponectin raised circulating adiponectin

Adiponectin plasma levels did not vary over the course of a saline infusion (2.6±0.2μg.mL^{-1} fasting vs. 2.3±0.1μg.mL^{-1} after 120min saline infusion). Infusion of 96μg.min^{-1}.kg^{-1} adiponectin for 60min increased circulating adiponectin levels

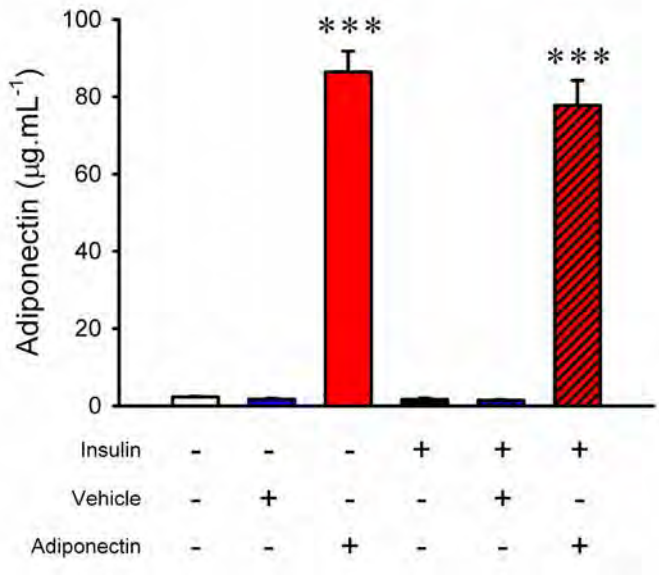

Figure 5.2: Plasma adiponectin concentrations. Samples were collected at *t*=120min; + designates infusion of indicated substance; insulin 3mU.min^{-1}.kg^{-1}, adiponectin 96μg.min^{-1}.kg^{-1}. *** Significantly different from saline (p<0.001; n=3-6).

approximately 40-fold (Figure 5.2). Neither 3mU.min^{-1}.kg^{-1} insulin nor vehicle infusion affected fasting plasma adiponectin. Insulin co-infusion slightly reduced the final plasma adiponectin concentration (77.8±6.4μg.mL^{-1} vs. 86.4±5.4μg.mL^{-1} with adiponectin alone; p<0.05), although it is unclear whether this difference would be biologically significant given the supra-physiological levels reached in both cases.

5.3.1.2 Effect of high-dose adiponectin on in vivo hemodynamics

Sampling procedures for the measurement of 2-deoxyglucose uptake required the arterial pressure monitor to be detached for the final 10min of the experiment, so no MAP or HR data were recorded after this time. Similarly, vascular resistance estimates after this point were excluded due to the reliance on MAP for their calculation. There were no relevant significant differences in MAP or HR between saline and insulin treatments. Adiponectin did not affect MAP or HR, either alone or in the presence of insulin (Figure 5.3).

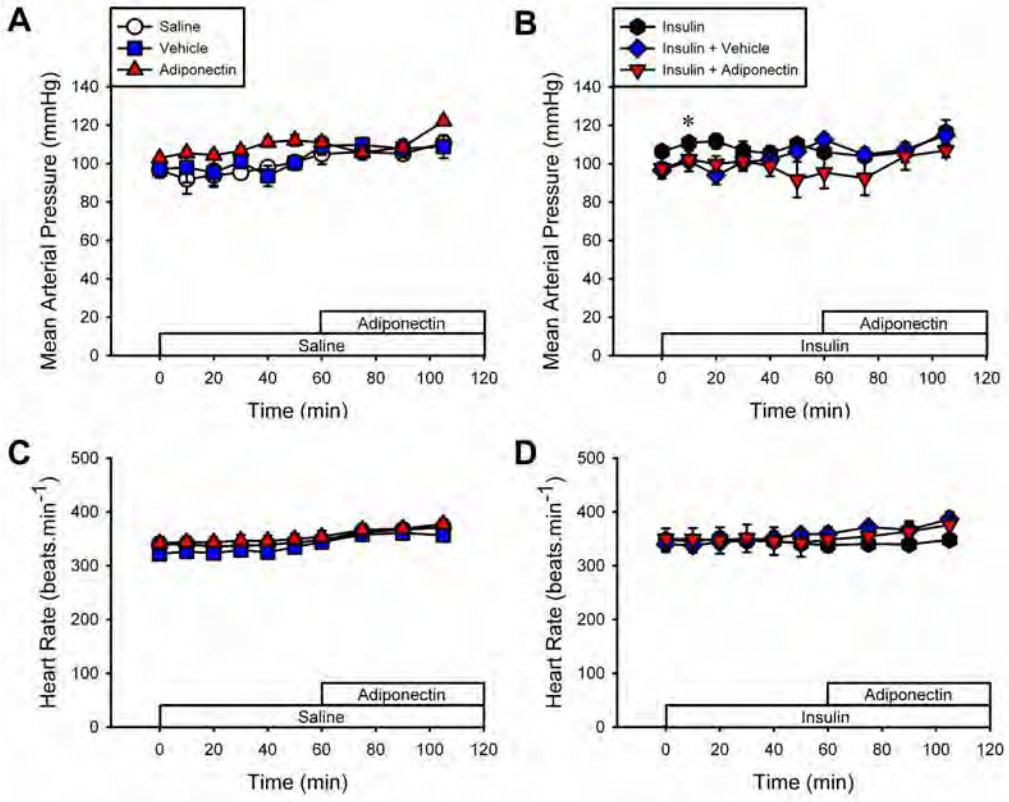

Figure 5.3: Adiponectin does not affect mean arterial pressure or heart rate. Upper: Time course of mean arterial pressure during vehicle or adiponectin infusion in the absence (A) or presence or 3mU.min^{-1}.kg^{-1} insulin (B). Lower: Time course of heart rate during vehicle or adiponectin infusion in the absence (C) or presence or 3mU.min^{-1}.kg^{-1} insulin (D). Mean arterial pressure and heart rate were monitored via a cannula placed in the carotid artery. * Significantly different from saline (p<0.05; n=5-7).

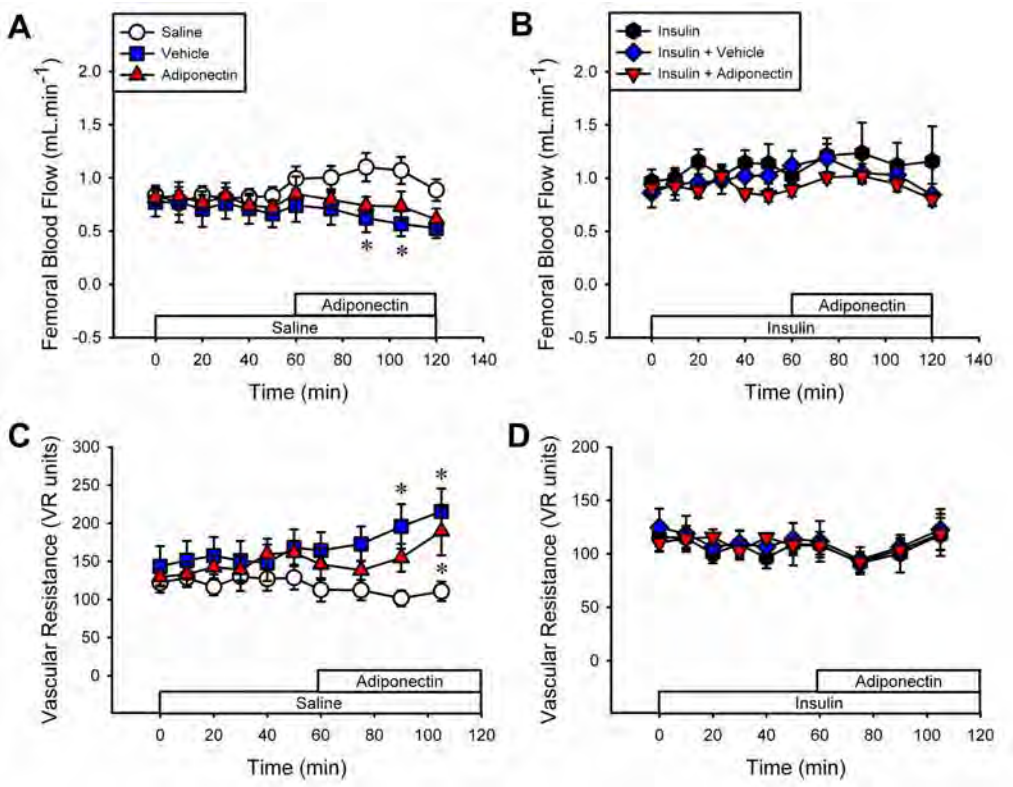

Figure 5.4: Femoral blood flow and vascular resistance. Upper: Time course of femoral blood flow during vehicle or adiponectin infusion in the absence (A) or presence or 3mU.min^{-1}.kg^{-1} insulin (B). Blood flow was measured by a transonic flow probe positioned around the femoral artery. Lower: Time course of vascular resistance during vehicle or adiponectin infusion in the absence (C) or presence or 3mU.min^{-1}.kg^{-1} insulin (D). Vascular resistance was calculated by dividing mean arterial pressure by femoral blood flow for each time point. * Significantly different from saline (p<0.05; n=5-7).

There was a trend for FBF to be reduced during adiponectin infusion, although this did not reach significance (0.7±0.1mL.min^{-1} vs. 1.1±0.1mL.min^{-1} after 90min saline infusion; p=0.069; Figure 5.4A). Consequently, there was a significant increase in VR after 45min of adiponectin infusion (Figure 5.4C). However, this cannot be solely attributed to adiponectin, as the same effect was demonstrated during vehicle infusion, with FBF significantly reduced and VR significantly increased by the vehicle within 30min (Figure 5.4A and C). The vehicle-induced changes were not apparent in the presence of insulin (Figure 5.4B and D). Whilst infusion of this relatively low dose of insulin was not found to significantly increase FBF, it is possible that a small, moderating effect of insulin is able to counteract the vehicle effects in these combined treatments.

Microvascular perfusion was assessed using the 1-MX metabolism technique (Rattigan *et al.*, 1997b). There was no significant difference in 1-MX metabolism between any of the groups, although it tended to be lower in rats treated with vehicle (Figure 5.5). Adiponectin appeared to counteract the effect of the vehicle, but further experiments would be required to confirm this.

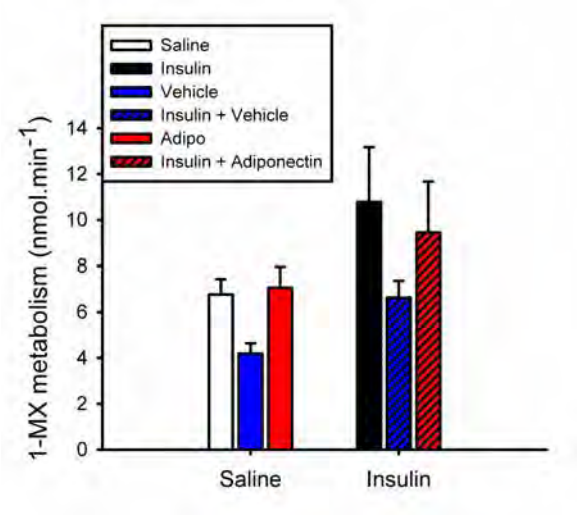

Figure 5.5: Microvascular perfusion. Effect of vehicle or adiponectin infusion in the presence or absence of 3mU.min^{-1}.kg^{-1} insulin on 1-methyxanthine metabolism. Plasma samples for analysis were collected at the completion of the experiment, *t*=120min (*n*=5-6).

5.3.1.3 Effect of adiponectin on glucose homeostasis

In contrast to expectations, adiponectin infusion in the absence of insulin increased blood glucose levels above that seen with saline ($p<0.01$ at 120min; Figure 5.6A). The same effect was apparent with vehicle infusion, suggesting that a bacterial contaminant rather than adiponectin was responsible. Thus, no glucose infusion was required during either treatment (Figure 5.6 A and C). However, in the presence of insulin, maintenance of constant blood glucose concentrations required infusion of exogenous glucose (Figure 5.6B and D). However, co-infusion of insulin with vehicle, but not adiponectin, required a significant reduction in the glucose infusion rate (GIR) to maintain stable blood glucose levels (Figure 5.6D).

To assess which tissues contributed to the change in GIR, hindleg glucose uptake (HGU), 2-deoxyglucose uptake (2-DG), rate of glucose appearance (Ra) and whole

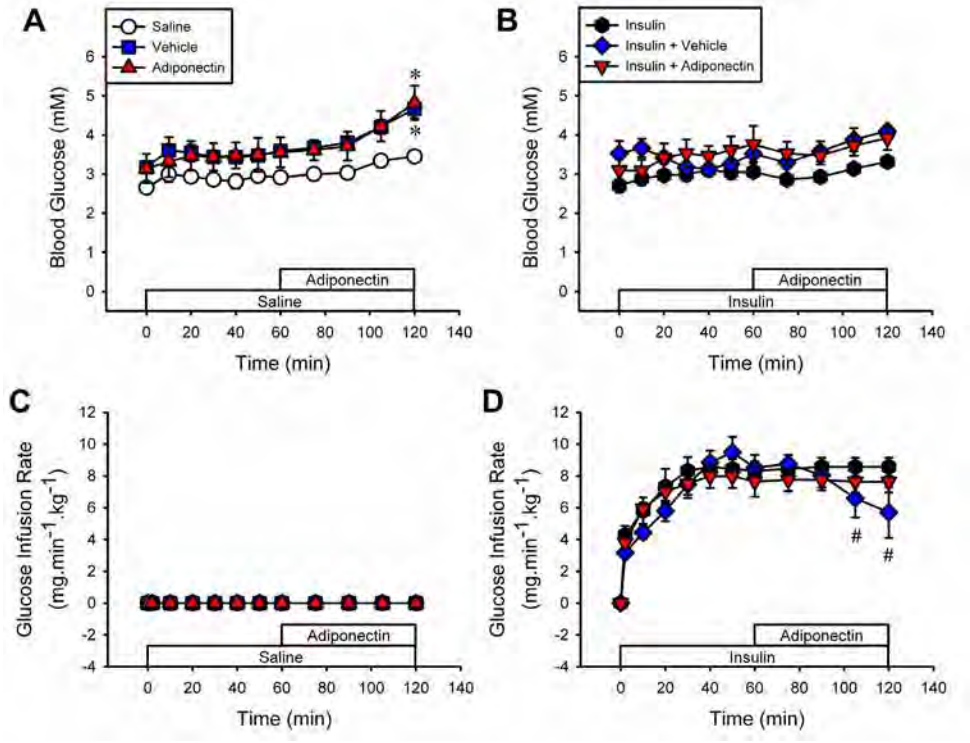

Figure 5.6: Blood glucose and glucose infusion rate. Upper: Time course of blood glucose concentrations during vehicle or adiponectin infusion in the absence (A) or presence or 3mU.min^{-1}.kg^{-1} insulin (B). Lower: Time course of glucose infusion rate during vehicle or adiponectin infusion in the absence (C) or presence or insulin (D). * Significantly different from saline and # significantly different from insulin (p<0.05; n=5-7).

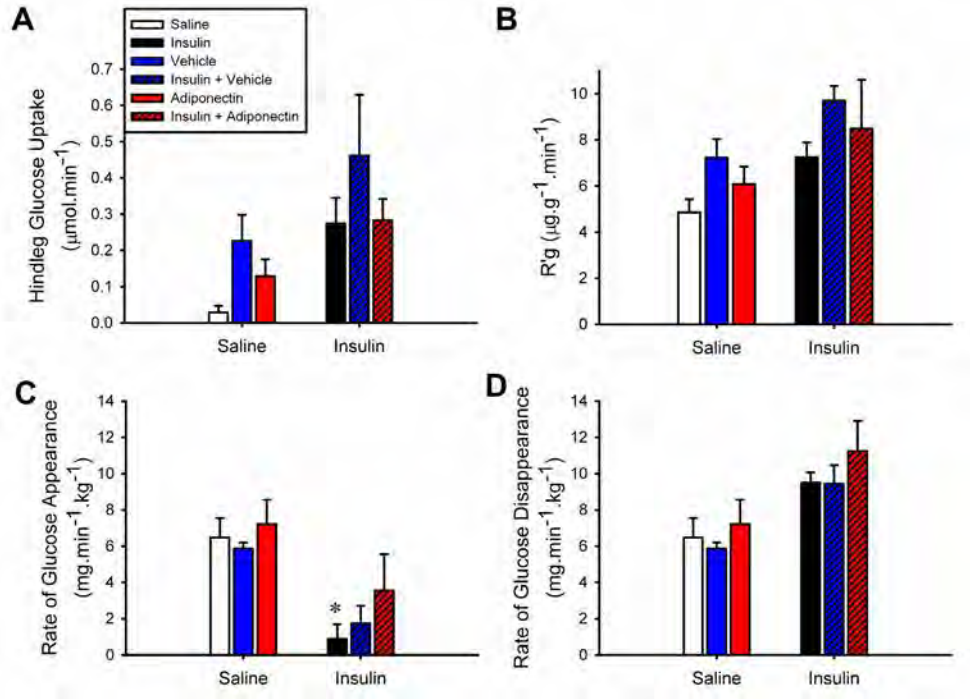

Figure 5.7: Glucose homeostasis. Effect of vehicle or adiponectin infusion in the presence or absence of 3mU.min^{-1}.kg^{-1} insulin on (A) hindleg glucose uptake, (B) radioactive 2-deoxy glucose uptake (R'g) into whole calf muscle, (C) the rate of glucose appearance (hepatic glucose output), and (D) the rate of glucose disappearance (whole body glucose uptake). All samples were collected at the completion of the experiment, t=120min. * Significantly different from saline (p<0.05; n=5-6).

body rate of glucose disposal (Rd) were measured during infusion of adiponectin or vehicle, alone or in the presence of insulin (Figure 5.7). Few of the changes reached significance, although adiponectin prevented the significant insulin-mediated reduction in Ra and vehicle treatment tended to increase HGU.

5.3.2 Effect of high-dose adiponectin in vivo in insulin resistance

The previous set of experiments did not show significant hemodynamic or metabolic actions of adiponectin. The interpretation of this data is inhibited by interference from effects of the bacterial vehicle preparation. However, the effect of the vehicle on 1-MX metabolism and GIR were not apparent in animals infused with the recombinant adiponectin preparation. This suggested that adiponectin may be augmenting microvascular perfusion and insulin sensitivity, and in this way counteracting the detrimental effects of the vehicle. As these adiponectin actions were small, it was hypothesised that vascular adiponectin action may be more apparent in insulin resistance, where the effects of insulin and endogenous adiponectin are likely to be diminished.

Table 5.2: Basal characteristics of normal chow and high-fat fed rats.

	Normal chow	High-fat	*p* value
Body weight (g)	232.6 ± 2.5	240.4 ± 3.0	0.051
Epididymal fat (g)	1.4 ± 0.1	3.0 ± 0.1	**<0.001**
Plasma glucose (mM)	6.0 ± 0.2	6.7 ± 0.2	**0.011**
Plasma insulin (pM)	108.4 ± 20.4	207.0 ± 22.2	**0.004**
Plasma adiponectin ($\mu g.mL^{-1}$)	2.1 ± 0.1	2.4 ± 0.1	0.140
Mean arterial pressure (mmHg)	100.1 ± 1.4	104.4 ± 1.3	**0.029**
Heart rate ($beats.min^{-1}$)	340.7 ± 4.8	366.4 ± 6.7	**0.002**
Femoral blood flow ($mL.min^{-1}$)	0.9 ± 0.0	0.7 ± 0.0	**0.008**
Vascular resistance (VR units)	123.5 ± 5.8	156.8 ± 7.9	**<0.001**

All measures taken at the commencement of the experiment, *t*=0min, except epididymal fat pads which were extracted upon experimental completion. Values are mean ± SE. *p* values as determined by Students *t* test (*n*=12-39).

5.3.2.1 High-fat feeding induces insulin resistance

Rats were fed a high-fat diet for four weeks to induce insulin resistance. Body weight of the high-fat fed rats was borderline significantly increased compared to normal-chow fed rats (Table 5.2). Epididymal fat pad weight was doubled following high-fat feeding, indicating a marked increase in adiposity. These data are consistent with previous findings from this thesis (Section 4.3.1.1) and other studies (St-Pierre *et al.*, 2010), demonstrating that the four week high-fat fed rat is a model of increased adiposity in the absence of overt obesity.

Fasting plasma concentrations of both glucose and insulin were significantly increased in high-fat fed rats (Table 5.2), in agreement with a previous study (St-Pierre *et al.*, 2010). In addition, glucose infusion rate ($4.7\pm0.0 mg.min^{-1}.kg^{-1}$ vs. $8.6\pm0.6 mg.min^{-1}.kg^{-1}$ in normal animals; $p<0.01$) and insulin-mediated suppression of hepatic glucose output ($4.9\pm0.9 mg.min^{-1}.kg^{-1}$ vs. $0.9\pm0.8 mg.min^{-1}.kg^{-1}$ in normal animals; $p<0.01$) were both markedly attenuated following high-fat feeding. These data demonstrate that rats fed a high-fat diet for four weeks develop insulin resistance. There was no significant change in fasting plasma adiponectin in response to the high-fat diet (Table 5.2). In conjunction with reduced insulin sensitivity, measures of macrovascular function were all significantly altered in high-fat fed rats (Table 5.2). However, the magnitudes of the hemodynamic changes were modest, and similar changes were not reported in a previous study (St-Pierre *et al.*, 2010), so it is unclear if they are biologically significant.

5.3.2.2 Effect of adiponectin on basal and insulin-mediated hemodynamics and metabolism in high-fat fed rats

Neither $96 \mu g.min^{-1}.kg^{-1}$ adiponectin nor vehicle infusion significantly altered any vascular parameter in high-fat fed rats, alone or in the presence of insulin (Table 5.3). However, there was a tendency for increased 1-MX metabolism in response to vehicle treatment (Table 5.3), in contrast to the effect of the vehicle in normal-chow fed animals (Figure 5.5). Muscle 2-deoxy glucose uptake was also not significantly affected by adiponectin or vehicle treatment, although there was a tendency for increased 2-DG uptake in vehicle treated rats (Table 5.3). Significant insulin-mediated

2-DG uptake was only apparent in the presence of vehicle or adiponectin ($p<0.05$ vs. saline).

Table 5.3: Adiponectin did not have vascular actions in high-fat fed rats.

	Saline			Insulin		
	Saline	Vehicle	Adiponectin	Saline	Vehicle	Adiponectin
Mean arterial pressure (mmHg)	110.4 ± 3.4	100.4 ± 7.6	104.0 ± 7.6	115.9 ± 4.1	87.1 ± 6.8	86.9 ± 8.8
Heart rate (beats.min-1)	358.5 ± 5.0	400.0 ± 4.8	388.4 ± 18.3	379.7 ± 13.8	383.8 ± 18.4	373.3 ± 19.5
Femoral blood flow (mL.min-1)	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.2
Vascular resistance (VR units)	102.8 ± 9.9	166.3 ± 19.6	135.5 ± 12.7	120.6 ± 20.5	90.1 ± 10.4	151.2 ± 25.5
1-MX metabolism (nmol.min-1)	4.5 ± 0.5	7.0 ± 1.3	7.4 ± 1.0	6.6 ± 0.3	9.7 ± 2.0	7.5 ± 1.5
2-deoxy glucose uptake (R'g)	4.9 ± 0.3	8.6 ± 0.7	7.2 ± 0.9	7.6 ± 0.6	9.2 ± 1.4 *	10.1 ± 0.7 *

All measures taken at the completion of the experiment, *t*=120min, except mean arterial pressure, heart rate and vascular resistance *t*=105min. Values are mean ± SE. * Significantly different from the corresponding treatment in the absence of insulin (*n*=4-7).

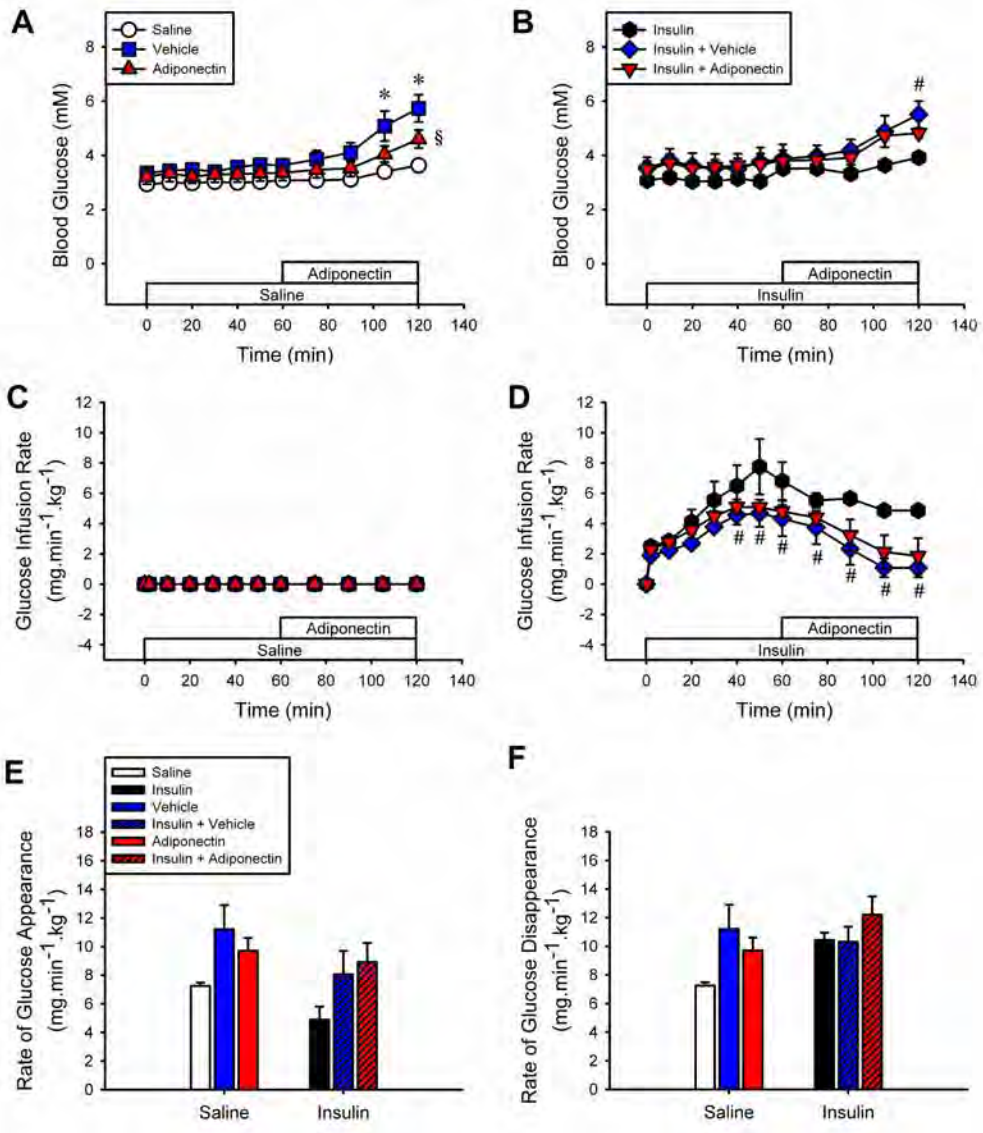

Figure 5.8: Adiponectin did not affect glucose metabolism in high-fat fed rats. Upper: Time course of blood glucose during vehicle or adiponectin infusion in the absence (A) or presence or 3mU.min^{-1}.kg^{-1} insulin (B). Middle: Time course of glucose infusion rate during vehicle or adiponectin infusion in the absence (C) or presence or insulin (D). Lower: Effect of vehicle or adiponectin infusion in the presence or absence of insulin on (E) the rate of glucose appearance (hepatic glucose output), and (F) the rate of glucose disappearance (whole body glucose uptake). Samples were collected at *t*=120min. * Significantly different from saline, # significantly different from insulin and § significantly different from vehicle ($p<0.05$; n=4-7).

As in the normal animals, blood glucose was significantly increased by vehicle infusion alone, in the absence of exogenous glucose infusion (Figure 5.8 A and C). A trend for increased blood glucose was also apparent in rats treated with adiponectin (4.6±0.3mM vs. saline 3.6±0.0mM; p=0.068; Figure 5.8A). However, adiponectin significantly blunted the vehicle-induced increase in blood glucose in high-fat fed rats

(4.6±0.3mM vs. vehicle 5.7±0.5mM at 120min; $p<0.05$). During insulin infusion, the glucose infusion rate was markedly reduced by co-infusion of either adiponectin or vehicle (Figure 5.8D). Over this time, blood glucose increased in both treatment groups, becoming significant in the case of insulin plus vehicle infusion (Figure 5.8B), suggesting that the glucose infusion rate should actually have been further reduced. There was no significant difference in the rates of glucose appearance and disappearance between any of the treatments following high-fat feeding (Figure 5.8 E and F). Despite this, the rate of hepatic glucose production appeared to be slightly higher in rats treated with adiponectin or vehicle than corresponding controls, possibly accounting for the increase in blood glucose. However, the Ra and Rd determinations of glucose turnover are based on the assumption that blood glucose concentrations are at steady-state. Therefore, these measurements may not be accurate under the current conditions of changing blood glucose.

Plasma adiponectin levels were not reduced following high-fat feeding, in contrast to previous reports (Yamauchi *et al.*, 2001; Mullen *et al.*, 2009). However, circulating adiponectin concentrations in the normal chow-fed Hooded Wistar rats used in this experiment were at the low end of the described range for adiponectin (Table 5.2). This, in conjunction with the lack of significant effect of insulin in these animals suggested that the insulin sensitivity may have already been reduced. Fasting plasma adiponectin was significantly higher in normal-chow fed Sprague-Dawley (Figure 4.4) than Hooded Wistar rats (7.5±0.5μg.mL^{-1} vs. 2.1±0.1μg.mL^{-1}, $p<0.001$). While both the hemodynamic and metabolic responses of the two strains are qualitatively similar, Sprague-Dawley rats may have a slightly higher muscle mass and be slightly more insulin sensitive than this population of Hooded Wistar rats (unpublished observation). Lowered sensitivity to insulin, and potentially also adiponectin (see section 4.1), may affect the results of the current studies. Therefore, further experiments were undertaken using Sprague-Dawley rats.

5.3.3 Pre-treatment and co-infusion of adiponectin during an insulin clamp revealed marked vehicle effects

In addition to indications in the normal-chow fed animals that adiponectin may counter the effects of the vehicle to improve 1-MX metabolism and GIR, I have shown significant adiponectin-mediated blunting of the vehicle-induced increase in basal blood glucose in high-fat fed rats. These data are suggestive that adiponectin may have beneficial vascular and metabolic actions, although this could not be confirmed. However, the results are confounded by substantial vehicle effects, in the presence of which the actions of adiponectin cannot be accurately assessed. Therefore, even with further experiments, definitive conclusions about the actions of adiponectin could not be drawn while the vehicle effects remained. Blood glucose levels did not rise significantly until after the vehicle was infused for at least 45min, suggesting the vehicle effects may be due to accumulation of a bacterial contaminant. To investigate whether this was the case, the protein preparations were infused earlier in the experiment. Additionally, it was previously shown that adiponectin opposes endothelin-1 action in the perfused rat hindlimb (Section 3.3.2.1). Based on reports that insulin stimulates production of ET-1 (Ferri *et al.*, 1995; Ferri *et al.*, 1996), it was hypothesised that adiponectin may be more effective at improving insulin sensitivity when present before insulin, and thus the increased ET-1. Therefore, adiponectin action was investigated using an adjusted experimental protocol to examine this hypothesis (Figure 5.1C).

Initial experiments exposed a dramatic drop in mean arterial pressure when 96μg.min^{-1}.kg^{-1} adiponectin was infused prior to and during insulin infusion (Figure 5.9A). The same response was apparent towards the end of a 60min infusion of the vehicle preparation (Figure 5.9B), indicating that this is a side-effect of the bacterial vehicle. Blood pressure recovered after the vehicle or adiponectin infusion ceased. These severe side effects of the vehicle were not apparent in the previous set of experiments, although the same dose was used in both cases. It is likely that these effects are the result of vehicle contaminants accumulating in the blood stream, as they were only apparent towards the end of the infusion. Therefore, it is probable that they were not apparent in the previous experiments because they occurred in the final minutes of the protocol, when mean arterial pressure and heart rate data were not available due to

procedural requirements. Alternatively, these side effects may have been limited by the increased presence of insulin due to prior infusion. Such side effects of the vehicle preparation affect the ability to assess adiponectin action, and may possibly counteract any effects of adiponectin. Thus it was considered vital to reduce the effects of the vehicle in order to accurately assess adiponectin action.

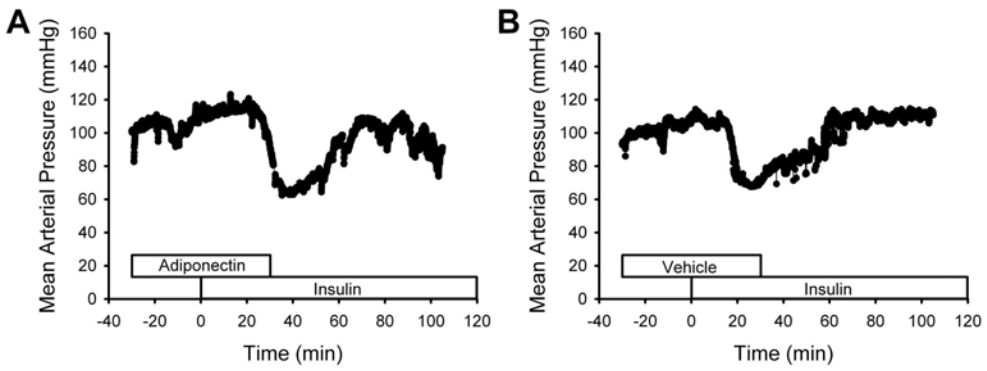

Figure 5.9: Early adiponectin infusion exposes marked vehicle effects. Representative trace of mean arterial pressure during (A) adiponectin or (B) vehicle pre-treatment and co-infusion with 3mU.min^{-1}.kg^{-1} insulin according to Figure 5.1C. Mean arterial pressure was monitored via a cannula placed in the carotid artery. Sampling artifacts have been excluded.

As the side effects of the vehicle appeared to be due to accumulation of contaminants, it was hypothesised that they may be sufficiently reduced by lowering the dose of infused adiponectin. Although adiponectin infusion was stopped 90min prior to the end of the experiment, plasma concentrations remained significantly elevated until completion of the protocol (Figure 5.10A). Plasma adiponectin was raised much higher than initially calculated for, as it has been recently been reported that adiponectin has a relatively long half-life (Halberg *et al.*, 2009). The approximately 40-fold increase in circulating adiponectin achieved with this infusion regime is clearly supra-physiological. Indeed, as the adiponectin assay used to produce these figures measures both LMW and HMW adiponectin multimers, and the highest proportion of endogenous adiponectin circulates as HMW (Waki *et al.*, 2003), the fold increase in the LMW adiponectin would actually be even higher. Therefore, it was concluded that plasma adiponectin would still be adequately increased using a significantly lower dose. This was shown; when the adiponectin dose was reduced by 75%, plasma adiponectin was increased approximately 4-fold (Figure 5.10B).

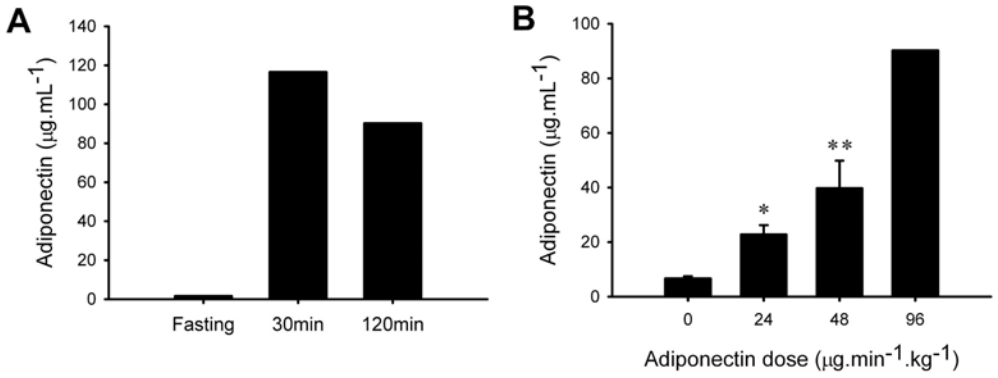

Figure 5.10: (A) Plasma adiponectin concentrations prior to and upon cessation of adiponectin infusion, and at completion of experiments conducted according to Figure 5.1C. (*n*=1). (B) Plasma adiponectin concentrations in response to varying adiponectin doses. Samples were collected at experimental completion, *t*=120min according to Figure 5.1C. * p<0.05, ** p<0.01, significantly different from 0μg.min^{-1}.kg^{-1} adiponectin (*n*=1-4).

5.3.4 Eliminating the vascular vehicle effects using a reduced dose did not expose adiponectin action

5.3.4.1 Adiponectin does not alter hemodynamics in vivo

Insulin significantly increased all measures of glucose uptake in Sprague-Dawley rats (glucose infusion rate: 6.7±0.5mg.min^{-1}.kg^{-1} vs. saline 0.0±0.0mg.min^{-1}.kg^{-1}; rate of glucose disappearance: 8.8±0.7mg.min^{-1}.kg^{-1} vs. saline 6.3±0.4mg.min^{-1}.kg^{-1}; hindleg glucose uptake: 0.32±0.03μmol.min^{-1} vs. saline 0.2±0.02μmol.min^{-1}; and 2-deoxy glucose uptake: 10.5±2.2μg.g^{-1}.min^{-1} vs. saline 2.2±0.2μg.g^{-1}.min^{-1}; p<0.05). Insulin also significantly decreased the rate of glucose appearance (1.7±0.4mg.min^{-1}.kg^{-1} vs. saline 6.3±0.4mg.min^{-1}.kg^{-1}; p<0.001). 1-MX metabolism tended to be higher in the presence of insulin compared to saline, although this did not reach significance (6.48±1.08nmol.min^{-1} vs. saline 4.6±0.6nmol.min^{-1}, n=7, p=0.28).

At the lower dose vehicle infusion, the effects on mean arterial pressure and heart rate seen at the higher dose were eliminated. Heart rate was significantly higher at all time points in the vehicle + insulin group than in rats treated with insulin alone, but neither group changed significantly from basal. There was also no change from insulin in femoral blood flow and vascular resistance with vehicle co-infusion (Figure 5.11).

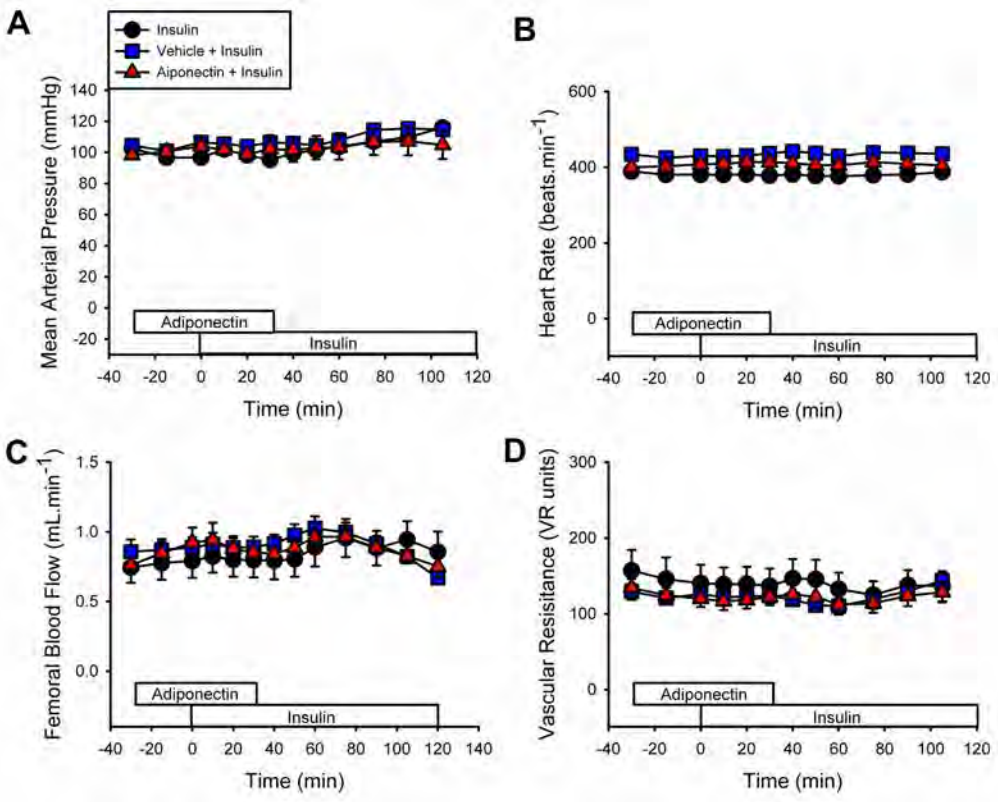

Figure 5.11: Adiponectin does not alter macrovascular parameters. Time course of (A) mean arterial pressure, (B) heart rate, (C) femoral blood flow, and (D) vascular resistance during vehicle or adiponectin pre-treatment and co-infusion with 3mU.min^{-1}.kg^{-1} insulin according to Figure 5.1C. Mean arterial pressure and heart rate were monitored via a cannula placed in the carotid artery. Blood flow was measured by a transonic flow probe positioned around the femoral artery. Vascular resistance was calculated by dividing mean arterial pressure by femoral blood flow for each time point. (*n*=6-8).

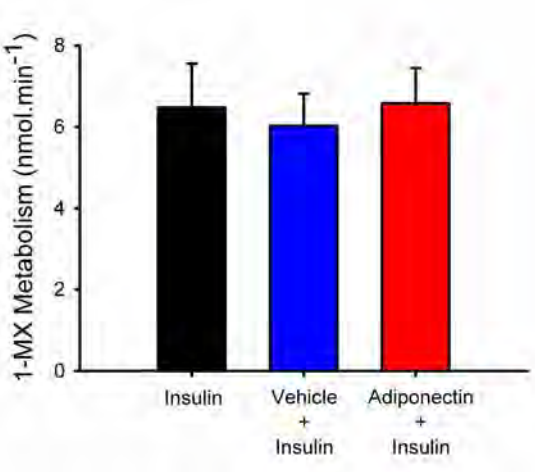

Figure 5.12: Adiponectin does not affect microvascular perfusion. Effect of vehicle or adiponectin pre-treatment and co-infusion with 3mU.min^{-1}.kg^{-1} insulin, on 1-methyxanthine metabolism. Plasma samples for analysis were collected at *t*=120min according to Figure 5.1C (*n*=6-7).

This further suggests that using a lower dose may avoid the effects of the vehicle on these measures, seen in the initial set of experiments using protocol A (Figure 5.2A). However, in the absence of hemodynamic vehicle effects, no action of adiponectin was revealed, with no significant difference from control treatment in any of the macrovascular measures. Insulin-stimulated microvascular perfusion was similarly unaffected by either vehicle or adiponectin infusion (Figure 5.12).

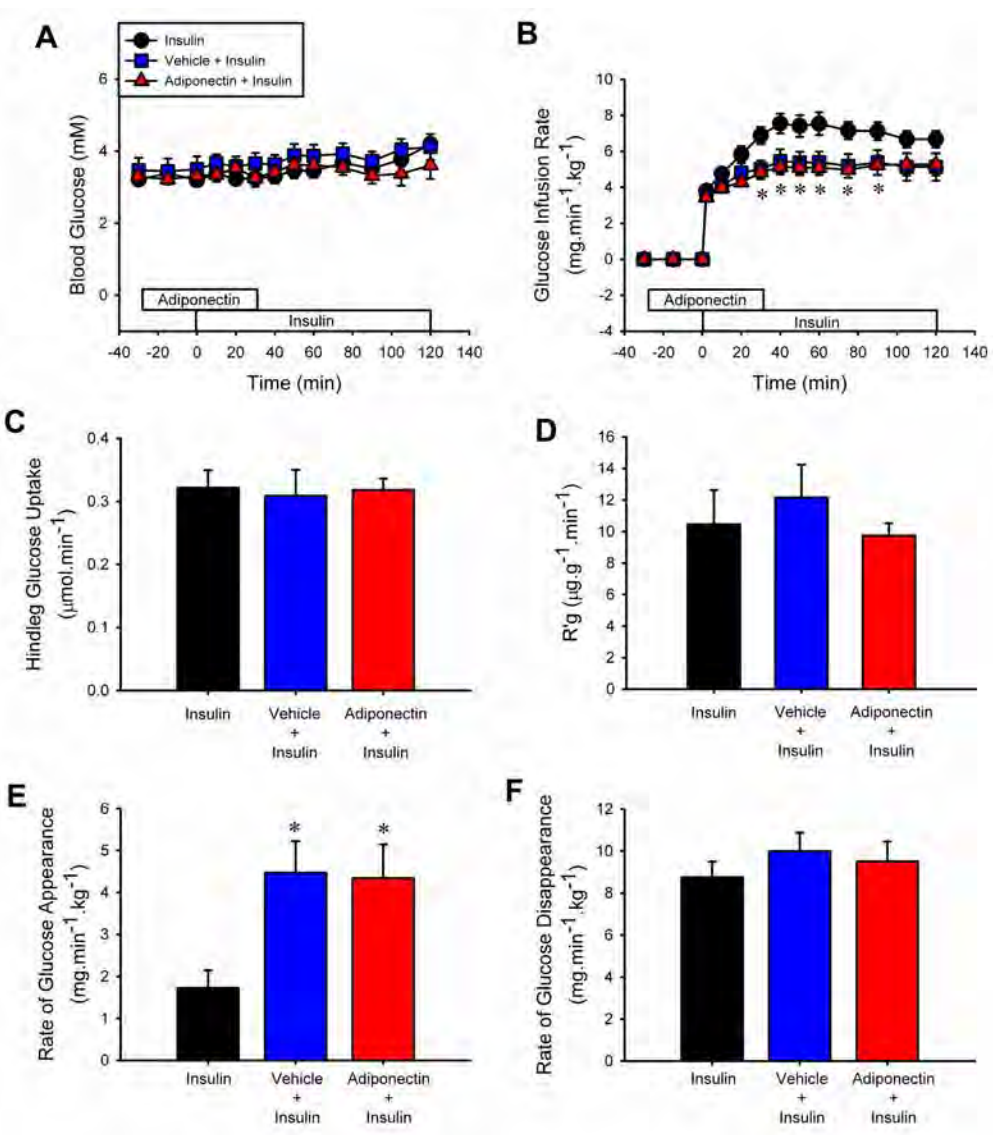

Figure 5.13: Adiponectin does not alter glucose homeostasis. Upper: Time course of (A) blood glucose, and (B) glucose infusion rate during vehicle or adiponectin pre-treatment and co-infusion with 3mU.min^{-1}.kg^{-1} insulin. Lower: Effect of vehicle or adiponectin pre-treatment and co-infusion with 3mU.min^{-1}.kg^{-1} insulin on (C) hindleg glucose uptake, (D) radioactive 2-deoxy glucose uptake (R'g) into whole calf muscle, (E) the rate of glucose appearance (hepatic glucose output), and (F) the rate of glucose disappearance (whole body glucose uptake). All samples were collected at the completion of the experiment, t=120min according to Figure 5.1C. * Significantly different from insulin (p<0.05; n=6-8).

5.3.4.2 Adiponectin does not alter insulin sensitivity

GIR was significantly reduced by infusion of vehicle between 30min and 90min, and tended to remain lowered until the end of the experiment (5.1±0.8mg.min^{-1}.kg^{-1} vs. insulin 6.7±0.5mg.min^{-1}.kg^{-1} at *t*=120min; *p*=0.077; Figure 5.13B). This may be attributable to a persistent effect of the vehicle to diminish insulin-mediated suppression of hepatic glucose output (Figure 5.13E). An increased rate of glucose appearance is also likely to be the cause of increased blood glucose and reduced GIR seen with the earlier protocols. However, reducing the dose of adiponectin, and thus vehicle, appears to have avoided the vehicle effects on 2-DG uptake, Rd and HGU (Figure 5.13). Adiponectin did not alter insulin-mediated 2-DG uptake or HGU, nor did it oppose the effects of the vehicle on Ra and GIR (Figure 5.13). Therefore, there was no indication that adiponectin improves insulin sensitivity *in vivo* acutely.

5.3.4.3 Adiponectin did not affect circulating levels of insulin or endothelin-1

Infusion of physiological insulin significantly increased fasting plasma insulin concentrations (Figure 5.14). Adiponectin infusion did not affect the circulating level of insulin achieved at completion of insulin infusion (Figure 5.14). This finding was consistent in the initial protocol using a higher adiponectin dose (Insulin: 361±65pM, Insulin + vehicle: 298±19pM, and Insulin + Adiponectin: 330±84pM; NS). Therefore, the absence of differences between groups in vascular and insulin sensitivity measures

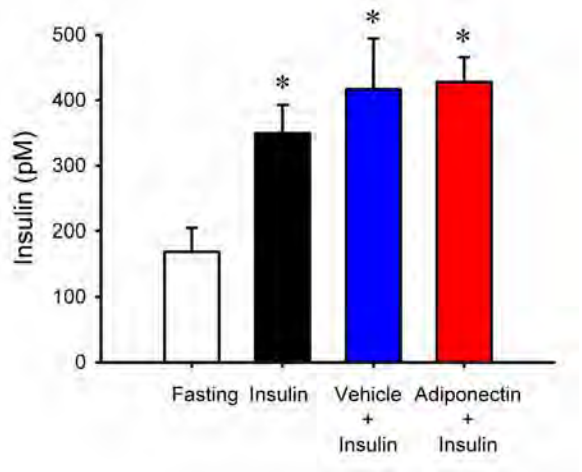

Figure 5.14: Adiponectin does not affect plasma insulin concentration. Effect of vehicle or adiponectin pre-treatment and co-infusion with 3mU.min^{-1}.kg^{-1} insulin on plasma insulin. Plasma samples for ELISA analysis were collected at *t*=120min according to Figure 5.1C. * Significantly different from fasting (*p*<0.05; *n*=6-19).

was not due to an altered effective dose of insulin.

The latter experimental protocol was designed on the premise that adiponectin may oppose the effects of insulin-stimulated ET-1 production. Neither adiponectin (4.2±0.6pM vs. 2.9±0.2pM with insulin alone after 120min; NS) nor vehicle (4.9±0.9pM vs. 2.9±0.2pM with insulin alone after 120min; NS) significantly altered plasma ET-1 levels at any dose. However, there was also no change in circulating ET-1 levels with insulin infusion (2.9±0.2pM vs. 2.0±0.5pM basally; NS), although changes in local ET-1 levels cannot be excluded. A previous study from our laboratory has described increased plasma ET-1 during a supra-physiological 10mU.min^{-1}.kg^{-1} insulin infusion (Ross *et al.*, 2007). Thus it seems likely that the physiological hyperinsulinemia used in the present study is not sufficient to stimulate endothelin-1 secretion at a detectable level. The hypothesis that adiponectin may improve insulin sensitivity by opposing endothelin-1 action *in vivo*, in a similar way to that shown in the perfused rat hindlimb, may be better investigated using a higher insulin dose. However, with increased insulin action, the likelihood of detecting an additional significant effect of adiponectin is reduced, making higher insulin dosage incompatible with the primary study aims.

5.4 Discussion

The acute hemodynamic and metabolic actions of adiponectin were examined in anaesthetised rats. No significant difference between adiponectin and vehicle groups was observed in any of the parameters measured, despite multiple protocols and doses being assessed. Specifically, adiponectin did not stimulate glucose uptake or microvascular perfusion, alone or in conjunction with insulin. Furthermore, there was no indication that acute adiponectin treatment attenuates insulin resistance.

The interpretation of this data is limited by confounding effects of a vehicle preparation purified from bacterial lysates. It is possible that the effects of the vehicle are counteracting, and thus masking, adiponectin action. A high dose infusion of vehicle alone reduced femoral blood flow, leading to increased vascular resistance, and increased blood glucose concentrations. In the presence of insulin, the vehicle blunted insulin-mediated suppression of hepatic glucose output causing a reduction in glucose infusion rate. At high doses, vehicle infusion also caused a dramatic drop in mean arterial pressure. Although endotoxin was not measured in the vehicle solutions, the effects observed are consistent endotoxic shock. The bacterial endotoxin lipopolysaccharide (LPS) stimulates the sympathetic nervous system causing blood pressure to drop (Vayssettes-Courchay *et al.*, 2005), which is consistent with the macrovascular vehicle effects observed at high doses. Endotoxins, alone and in conjunction with pro-inflammatory cytokines, reduce insulin-stimulated glucose uptake (Ling *et al.*, 1994). Similar combined treatment also leads to induction of iNOS (Kapur *et al.*, 1999; Bédard *et al.*, 1997), which has been shown to reduce insulin sensitivity, most notably by increasing hepatic glucose production basally and following insulin stimulation (Charbonneau & Marette, 2010). Furthermore, it has been directly demonstrated that LPS raises hepatic glucose output (Sugita *et al.*, 2002). Sugita *et al.* (2002) also showed increased blood glucose and dramatically impaired insulin-stimulated GIR within 60min of LPS treatment, indicating that such endotoxin effects are highly relevant to the current acute study. These data complement the present observations of a persistent effect of the bacterial vehicle preparation on the rate of glucose appearance. In addition, increased iNOS activity in

conjunction with augmented iNOS in insulin resistance, may explain the apparent tendency of the vehicle to increase glucose uptake and microvascular perfusion in high-fat fed rats. Such effects may also be more apparent following high-fat feeding as the compensatory actions of insulin are diminished. Globular adiponectin has been shown to reduce the inflammatory effects of bacterially-derived LPS (Noriaki *et al.*, 2009), possibly accounting for blunted vehicle effects on glucose homeostasis in some of the adiponectin treated groups. However, that study looked at changes in mRNA expression and protein levels after 12h of adiponectin exposure, and so may not be relevant to the 60min treatment used here (Noriaki *et al.*, 2009). Alternatively, the small but significant effect of adiponectin on blood glucose in the high-fat fed rats may be due a minor but independent action of adiponectin on glucose homeostasis counteracting the vehicle effects. Overall, these data demonstrate that experimental results obtained using bacterially produced proteins should be interpreted with caution, and in particular, emphasise the importance of an appropriate vehicle control.

The vehicle preparation used in the present studies may not accurately represent the contaminants in the adiponectin preparation. Comparison of the measured adiponectin and total protein concentrations of the recombinant adiponectin and vehicle preparations suggests that the vehicle may actually contain more contaminants than the adiponectin preparation, although the potential inaccuracy of these measures has been discussed in Chapter 2. It is possible that the large amounts of recombinant protein present in the adiponectin solution compete with bacterial contaminants to bind to the affinity purification column, resulting in higher levels of contaminants in the vehicle preparation. This may explain the apparent adiponectin-mediated improvements in some of the vehicle side-effects. Use of bacteria expressing an inactive scramble protein sequence for production of a vehicle preparation would better control for the possibility of this occurring. However, the nature of the vehicle effects are consistent with the presence of an endotoxin such as LPS, which is unlikely to be affected by competitive protein binding. Products are available for the detection and removal of these endotoxins from solutions, although for the purposes of *in vivo* studies it is important to consider any effects that the added purification procedure may have on protein yield. Further consideration of the preparation of infusion solutions may improve interpretation of future physiological studies using bacterially

expressed recombinant proteins. The current study cannot definitively conclude a lack of adiponectin effects in the absence of complete elimination of vehicle effects.

A number of studies have previously described reduced endogenous glucose production in response to adiponectin. However, vehicle effects aside, this finding was not replicated in the present analysis. Plasma adiponectin concentrations in humans are negatively correlated with basal and insulin-suppressed endogenous glucose production, independent of glucose tolerance (Stefan *et al.*, 2003). Combs *et al.* (2001) found that constant infusion of mammalian full-length adiponectin in the presence of insulin in fasted, conscious mice elicited a 73% increase in glucose infusion rate. There was no difference in whole body glucose disposal; rather the improved insulin sensitivity was solely attributable to a significant suppression of hepatic glucose production (Combs *et al.*, 2001). In another report from the same laboratory using an identical adiponectin preparation, Berg *et al.* (2001) observed an acute effect of mammalian adiponectin to reduce basal blood glucose that was not apparent using bacterial globular adiponectin. Post-translationally modified, mammalian adiponectin is also dramatically more effective at enhancing insulin-mediated inhibition of glucose production from isolated hepatocytes than preparations limited to LMW multimers (Wang *et al.*, 2002). Thus, the lack of adiponectin-mediated suppression of hepatic glucose output in the current study can be attributed to the LMW adiponectin preparation used, as all the above described studies demonstrated effects using HMW adiponectin. This is not entirely surprising, since it has previously been established that HMW adiponectin multimers preferentially act in the liver, as discussed in Chapter 2. However, the predominantly LMW bacterial adiponectin preparation was specifically chosen for the present work to evaluate adiponectin action in skeletal muscle and its associated vasculature. Therefore, these discrepant results do not detract from the conclusions of the current study, but rather support the finding that LMW adiponectin does not display significant hepatic activity.

The results of the various experiments comprising this work indicate that adiponectin does not have significant acute hemodynamic or metabolic effects *in vivo*, within the limits of the measurement system. It is possible that beneficial actions of adiponectin

may have become apparent in the absence of confounding vehicle effects. However, the broad-scale absence of significant differences between adiponectin and vehicle treated groups, in both normal and insulin resistant animals, tends to indicate that adiponectin is genuinely not having any effect. The lack of response is unlikely to result from insufficient adiponectin dosage, as the plasma concentrations achieved are substantially higher than physiological levels, with up to 40-fold elevations. The adiponectin dose was reduced in order to eliminate the majority of vehicle effects, but even under these conditions adiponectin action was not apparent. Using a lower dose again raises the possibility that effects may be being missed. However, even the lowest dose evaluated elicited a four-fold increase in plasma adiponectin. Significant improvements in *in vivo* insulin sensitivity have been demonstrated with only an approximate doubling of plasma adiponectin (Liu *et al.*, 2009a; Combs *et al.*, 2001). The primary aim of this work was to examine the acute effect of adiponectin in the *in vivo* vasculature, and even with adiponectin increased four-fold and all vascular vehicle effects abolished, there was no detectable adiponectin-mediated change in any vascular measure. Therefore, it is unlikely that bacterial contaminants are preventing an adiponectin effect from being observed. In addition, the finding of Combs *et al.* (2001) that the insulin-sensitising effect of HMW adiponectin is entirely attributable to hepatic actions suggests it does not have contributing hemodynamic effects, and thus such effects have not been missed in the present study due to the choice of adiponectin preparation. This study was not able to address the question of whether adiponectin resistance occurs in insulin resistance due to the lack of adiponectin activity in both normal-chow and high-fat fed animals, although I have shown that this model of mild insulin resistance is not adiponectin-deficient. The limited vascular effects of adiponectin previously observed in isolated vessels and my experiments in the perfused rat hindlimb did not translate into detectable augmentation of vascular parameters *in vivo*. It has previously been reported that adiponectin-mediated activation of eNOS plateaus after around an hour and at a dose of approximately 10ug.mL^{-1} (Hattori *et al.*, 2008). Therefore, *in vivo*, vascular adiponectin signalling pathways may already be maximally activated by endogenous adiponectin. Additionally, there are a wide range of vascular influences *in vivo*, and a moderate effect of adiponectin may not be distinguishable in this setting. Therefore, contributions to hemodynamic regulation do not appear to be a major aspect of adiponectin action *in vivo*.

Hemodynamic and metabolic responses to adiponectin were assessed in the anaesthetised rat under a range of different conditions. There were no substantial differences between adiponectin and vehicle treatments in any measure, whether alone or in the presence of insulin. The absence of adiponectin action was demonstrated in both normal and insulin resistant animals. Therefore, I conclude that low molecular weight adiponectin does not acutely alter vascular action or muscle metabolism *in vivo*.

CHAPTER 6 – DISCUSSION

This thesis has examined the acute hemodynamic and metabolic actions of adiponectin. Full-length recombinant adiponectin was produced in bacteria, and was shown to consist predominantly of trimeric adiponectin. This adiponectin stimulated eNOS phosphorylation *in vitro*, suggesting it has similar biological activity to preparations described in the literature. The acute physiological response to this adiponectin preparation was examined in the perfused rat hindlimb, where it was observed to oppose the vasoconstrictor effects of ET-1. This finding suggests that vascular actions of adiponectin may make important contributions to its beneficial effects on both insulin sensitivity and cardiovascular health. In addition, the specificity of this acute action suggested that this effect was NO-independent, indicating a novel mechanism of adiponectin action. The acute adiponectin-mediated inhibition of ET-1 action was not apparent in rats fed a high-fat diet to induce insulin resistance, possibly pointing to concurrent adiponectin resistance. However, the vasoconstrictor response to ET-1 was also diminished following high-fat feeding, due to enhanced activity of iNOS. This suggests that interaction between increased levels of both ET-1 and iNOS under conditions of metabolic stress may contribute to the associated vascular dysfunction. The systemic, acute actions of adiponectin were then investigated in anaesthetised rats. Adiponectin had no significant effect on basal or insulin-stimulated measures of glucose homeostasis and hemodynamic variables in normal animals, within the limits of detection. Similarly, there was no significant improvement in glucose homeostasis or vascular measures in insulin resistant high-fat fed rats acutely treated with adiponectin. Therefore, it appears that acute vascular actions do not substantially contribute to adiponectin action *in vivo*.

The data included in this thesis was all acquired using bacterially produced adiponectin, which is limited to forming low molecular weight (LMW) multimers. However, different forms of adiponectin exhibit variable properties, which affect experimental findings. It is primarily high molecular weight (HMW) adiponectin levels that correlate to increased insulin sensitivity (Lara-Castro *et al.*, 2006; Pajvani *et al.*, 2004). HMW, but not LMW, adiponectin multimers are downregulated in diabetic individuals (Basu *et al.*, 2007), and HMW adiponectin is selectively increased

following weight loss (Kobayashi *et al.*, 2004). As described in Chapter 5, HMW adiponectin has action to inhibit endogenous glucose production that is not seen with the LMW form (Wang *et al.*, 2002; Berg *et al.*, 2001). In addition, chronic infusion of adiponectin purified from foetal bovine serum, but not protein that was bacterially expressed, improved insulin sensitivity in high-fat fed mice (Wang *et al.*, 2004). Therefore, it appears that the effects of adiponectin on whole-body insulin sensitivity are primarily attributable to HMW multimers.

It was hypothesised that LMW adiponectin may have beneficial effects on insulin sensitivity via acute muscle or vascular actions. However, the findings of this thesis suggest that such effects are not an important part of adiponectin action, a conclusion supported by previous studies. Improved insulin sensitivity following rosiglitazone treatment is associated with increases in AdipoR1 expression in adipose tissue, while levels in skeletal muscle are decreased (Tan *et al.*, 2005). Similarly, insulin signalling was impaired in the liver, but not skeletal muscle, of adiponectin knockout mice (Yano *et al.*, 2008). Furthermore, Halperin *et al.* (2005) reported that neither total nor HMW adiponectin concentrations correlated with endothelium-dependent vasodilation in individuals at risk of developing type 2 diabetes and cardiovascular disease.

This raises the question of whether LMW adiponectin has physiologically significant actions. Adiponectin has been reported in cerebrospinal fluid (CSF) at concentrations of approximately 1-4% of circulating levels (Qi *et al.*, 2004), and CSF adiponectin concentration correlates with serum adiponectin (Kusminski *et al.*, 2007). Intravenous injection of adiponectin increased both serum and CSF measures of adiponectin, and caused decreased body weight through increased energy expenditure in both normal and obese mice (Qi *et al.*, 2004). Full-length and globular adiponectin, and a mutated form of adiponectin unable to form HMW structures, were equally effective at eliciting these effects (Qi *et al.*, 2004). Additionally, trimeric adiponectin preferentially accumulates in CSF, while HMW adiponectin multimers are undetectable (Kusminski *et al.*, 2007). Adiponectin has also been found to act in the brain via AMPK to stimulate food intake (Kubota *et al.*, 2007). However, Spranger *et al.* (2006) were unable to detect adiponectin in CSF and found that adiponectin was unable to cross the blood-brain barrier. The authors alternatively suggested that

adiponectin may act via its receptors on brain endothelial cells (Spranger *et al.*, 2006). These data suggest that LMW adiponectin may be of primary importance in eliciting central effects.

Emerging evidence suggests that the primary role of adiponectin may actually be involved in lipid processing. While PPARγ is the most referenced promoter of adiponectin, the gene has a number of other transcriptional controls, including sterol regulatory element-binding protein (SREBP), which is associated with fatty acid metabolism, cholesterol homeostasis and adipogenesis (Schäffler *et al.*, 1998; Kim *et al.*, 2006). Adiponectin in turn suppresses expression of SREBP, leading to decreased fatty acid synthesis in the liver (Awazawa *et al.*, 2009). Adiponectin knockout mice have increased serum triglyceride concentrations (Kubota *et al.*, 2002), as well as less ability to clear fat from the blood stream following a challenge (Maeda *et al.*, 2002a). Meanwhile, clearance of plasma free fatty acids (FFAs) is accelerated by both acute adiponectin treatment and adiponectin overexpression (Combs *et al.*, 2004; Fruebis *et al.*, 2001; Wang *et al.*, 2004). In one study, lipid clearance was shown to be markedly improved by globular adiponectin, while the full-length protein was significantly less effective (Fruebis *et al.*, 2001). It has previously been shown that adiponectin-mediated improvements in insulin sensitivity are associated with decreased circulating FFA and triglyceride levels, and decreased triglyceride content in both muscle and liver (Fruebis *et al.*, 2001; Yamauchi *et al.*, 2001; Wang *et al.*, 2004). Therefore, it has been suggested that adiponectin may improve insulin sensitivity indirectly, via a reduction in intramuscular lipid accumulation (reviewed in Dyck *et al.*, 2006). Speculation that adiponectin opposes ET-1 vasoconstriction by affecting cholesterol handling, also supports a primary role of adiponectin in lipid processing.

Resistance to the vascular actions of insulin has been widely associated with increases in circulating lipids. Feeding animals a high-fat diet raises plasma FFA (Henriksen *et al.*, 2008; Bourgoin *et al.*, 2008). Isolated endothelial cells treated for three hours with FFA demonstrated impaired insulin signalling via Akt and eNOS, leading to decreased NO production (Kim *et al.*, 2005). Results from Symons *et al.* (2009) indicate that vascular dysfunction and hypertension following high-fat feeding may be due to FFA-mediated impairment of eNOS phosphorylation. Consistent with this is the proposal

that FFAs activate protein kinase C θ in endothelium, which inhibits insulin-mediated Akt activation and stimulates insulin-mediated ERK1/2 activation, resulting in a shift towards insulin-mediated, ET-1-dependent vasoconstriction (Bakker *et al.*, 2008). Infusion of intralipid into humans was found to impair basal NO release, as well as insulin-stimulated leg blood flow and whole body glucose disposal (Steinberg *et al.*, 2000). The change in glucose disposal rate with insulin was strongly correlated with insulin-stimulated leg blood flow, indicating that FFAs primarily affect vascular insulin sensitivity (Steinberg *et al.*, 2000). It has been suggested that local accumulation of perivascular adipose tissue may impair nutritive blood flow (reviewed in Eringa *et al.*, 2007). Acute elevations in plasma FFA impair insulin-mediated glucose uptake and microvascular perfusion in both rats and humans (Clerk *et al.*, 2002; Liu *et al.*, 2009b; de Jongh, 2004). The FFA-induced effects on insulin-stimulated glucose metabolism were time-dependent, occurring approximately 1.5 hours after FFA elevation (Clerk *et al.*, 2002). Therefore, if adiponectin improves insulin sensitivity largely by reducing FFA concentrations, a significant change would not be apparent during the 1 hour adiponectin infusion used in the current *in vivo* study.

In one of the most dramatic studies into adiponectin action, Kim *et al.* (2007) have shown that leptin knockout and adiponectin overexpressing crossover mice show no signs of insulin resistance, despite severe obesity. Adiponectin overexpression also prevented the development of further insulin resistance when these mice were challenged with a high-fat diet, despite an even greater weight gain. Although overall fat mass was increased, improved insulin sensitivity was associated with reductions in visceral fat. These mice also displayed decreased circulating triglycerides, improved triglyceride clearance after lipid challenge and an increased number of smaller adipocytes. The authors suggested that adiponectin promotes storage of triglycerides preferentially in adipose tissues, reducing the levels in liver and muscle and thus improving insulin sensitivity (Kim *et al.*, 2007). Conversely, the work by Mullen *et al.* (2009) describing adiponectin resistance preceding lipid accumulation and impaired insulin action in muscle, lead to the proposal that rapid loss of adiponectin-mediated fat oxidation leaves the muscle less able to respond to the excess lipid exposure during high-fat feeding. Visceral adipose tissue is especially detrimental to insulin sensitivity,

and adipocytes isolated from visceral fat are less sensitive to the antilipolytic effects of insulin, leading to increased FFA release (van Harmelen *et al.*, 2008). Rosiglitazone-mediated improvements in insulin sensitivity are associated with both increased adiponectin and adipose tissue remodelling, producing smaller adipocytes in which adiponectin secretion is greater (Dallaire *et al.*, 2008). In addition, this tissue remodelling means that treatment with thiazoledinediones is usually correlated with increased body weight gain, partially due to differentiation of fat cells (Dallaire *et al.*, 2008; Combs *et al.*, 2002). Furthermore, it has been proposed that adipose tissue is hypoxic in obesity, causing inflammation and leading to diabetes. Adiponectin promotes angiogenesis and development of more, smaller adipocytes, decreasing the diffusion distance, thus potentially improving oxygen availability in adipose tissue (Trayhurn *et al.*, 2008). Overall, these findings indicate that the most significant actions of adiponectin may be in lipid processing, by encouraging appropriate fat deposition and lowering circulating FFAs, thereby indirectly improving insulin sensitivity. Adiponectin-mediated modulation of lipid storage would require chronic exposure, and is thus consistent with the present findings that LMW adiponectin does not acutely alter metabolism or hemodynamic regulation *in vivo*. The conclusions of this thesis are in agreement with a recent proposal by Kadowaki *et al.* (2008) that adiponectin may act as a starvation signal, with HMW multimers redirecting fuel utilisation in peripheral tissue from glucose to fatty acids, and LMW multimers acting in the brain to increase food intake, decrease energy expenditure and encourage fat storage.

The aim of this thesis was to investigate whether adiponectin has acute vascular actions that contribute to its role as an insulin sensitiser. I have identified a novel effect of adiponectin to oppose ET-1-mediated vasoconstriction in an isolated vascular system. However, it appears that vascular effects of adiponectin may be of minor physiological significance, as adiponectin had no effect on acute hemodynamic or metabolic parameters *in vivo*. The long-term effects of adiponectin have not been examined here, and it is possible that adiponectin acts in the vasculature to improve insulin sensitivity chronically. It is proposed that the primary function of low molecular weight adiponectin is in lipid processing, which may in turn chronically modulate hemodynamic regulation.

REFERENCES

Alabadí JA, Miranda FJ, Lloréns S, Centeno JM, Marrachelli VG, Alborch E (2004). Mechanisms underlying diabetes enhancement of endothelin-1-induced contraction in rabbit basilar artery. *Eur. J. Pharmacol.* **486**(3)**:** 289-296.

Amiri F, Ko EA, Javeshghani D, Reudelhuber TL, Schiffrin EL (2010). Deleterious combined effects of salt-loading and endothelial cell restricted endothelin-1 overexpression on blood pressure and vascular function in mice. *J. Hypertens.* **28**(6)**:** 1243-1251.

Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J-i, Hotta K, Shimomura I, Nakamura T, Miyaoka K (1999). Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem. Biophys. Res. Commun.* **257**(1)**:** 79-83.

Asp S, Watkinson A, Oakes ND, Kraegen EW (1997). Prior eccentric contractions impair maximal insulin action on muscle glucose uptake in the conscious rat. *J. Appl. Physiol.* **82**(4)**:** 1327-1332.

Awazawa M, Ueki K, Inabe K, Yamauchi T, Kaneko K, Okazaki Y, Bardeesy N, Ohnishi S, Nagai R, Kadowaki T (2009). Adiponectin suppresses hepatic SREBP1c expression in an AdipoR1/LKB1/AMPK dependent pathway. *Biochem. Biophys. Res. Commun.* **382**(1)**:** 51-56.

Bakker W, Sipkema P, Stehouwer CDA, Serne EH, Smulders YM, van Hinsbergh VWM, Eringa EC (2008). Protein Kinase C Î¸ Activation Induces Insulin-Mediated Constriction of Muscle Resistance Arteries. *Diabetes* **57**(3)**:** 706-713.

Baron AD (1994). Hemodynamic actions of insulin. *Am J Physiol Endocrinol Metab* **267**(2)**:** E187-202.

Baron AD, Brechtel G (1993). Insulin differentially regulates systemic and skeletal muscle vascular resistance. *Am J Physiol Endocrinol Metab* **265**(1)**:** E61-67.

Baron AD, Clark MG (1997). Role of blood flow in the regulation of muscle glucose uptake. *Annu. Rev. Nutr.* **17**(1)**:** 487-499.

Barton M, Haudenschild CC, D'Uscio LV, Shaw S, Munter K, Luscher TF (1998). Endothelin ETA receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. *Proc. Natl. Acad. Sci. U. S. A.* **95**(24)**:** 14367-14372.

Basu R, Pajvani UB, Rizza RA, Scherer PE (2007). Selective downregulation of the high molecular weight form of adiponectin in hyperinsulinemia and in type 2 diabetes: Differential regulation from nondiabetic subjects. *Diabetes* **56**(8)**:** 2174-2177.

Bédard S, Marcotte B, Marette A (1997). Cytokines modulate glucose transport in skeletal muscle by inducing the expression of inducible nitric oxide synthase. *Biochem J.* **325**(Pt 2)**:** 487-493.

Berg A, Combs T, Du X, Brownlee M, Scherer P (2001). The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat. Med.* **7**(8)**:** 947-953.

Bergdahl A, Gomez MF, Dreja K, Xu S-Z, Adner M, Beech DJ, Broman J, Hellstrand P, Sward K (2003). Cholesterol Depletion Impairs Vascular Reactivity to Endothelin-1 by Reducing Store-Operated Ca2+ Entry Dependent on TRPC1. *Circ. Res.* **93**(9)**:** 839-847.

Bertani S, Carboni L, Criado A, Michielin F, Mangiarini L, Vicentini E (2010). Circadian profile of peripheral hormone levels in sprague-dawley rats and in common marmosets (Callithrix jacchus). *In Vivo* **24**(6)**:** 827-836.

Bluher M, Bullen JW Jr, Lee JH, Kralisch S, Fasshauer M, Kloting N, Niebauer J, Schon MR, Williams CJ, Mantzoros CS. (2006). Circulating adiponectin and expression of adiponectin receptors in human skeletal muscle: associations with metabolic parameters and insulin resistance and regulation by physical training. *J. Clin. Endocrinol. Metab.* **91**(6)**:** 2310-2316.

Bonadonna R, Del Prato S, Saccomani MP, Bonora E, Gulli G, Ferrannini E, Bier D, Cobelli C, DeFronzo RA (1993). Transmembrane glucose transport in skeletal muscle of patients with non-insulin-dependent diabetes. *J. Clin. Invest.* **92:** 486-494.

Bourgoin F, Bachelard H, Badeau M, Melancon S, Pitre M, Lariviere R, Nadeau A (2008). Endothelial and vascular dysfunctions and insulin resistance in rats fed a high-fat, high-sucrose diet. *Am J Physiol Heart Circ Physiol* **295**(3)**:** H1044-1055.

Bradley E, Ross R, Clark M, McConell G, Rattigan S (2007). Local L-NAME administration blocks insulin-mediated but not contraction-mediated capillary recruitment in rat hindleg muscles. *Diabetes* **56**(Suppl 1)**:** A397.

Bradley EA, Eringa EC, Stehouwer CDA, Korstjens I, van Nieuw Amerongen GP, Musters R, Sipkema P, Clark MG, Rattigan S (2010). Activation of AMP-Activated Protein Kinase by 5-Aminoimidazole-4-Carboxamide-1-β-D-Ribofuranoside in the

Muscle Microcirculation Increases Nitric Oxide Synthesis and Microvascular Perfusion. *Arterioscler. Thromb. Vasc. Biol.* **30**(6)**:** 1137-1142.

Bradley S, Kingwell B, McConell G (1999). Nitric oxide synthase inhibition reduces leg glucose uptake but not blood flow during dynamic exercise in humans. *Diabetes* **48**(9)**:** 1815-1821.

Bruce CR, Mertz VA, Heigenhauser GJF, Dyck DJ (2005). The stimulatory effect of globular adiponectin on insulin-stimulated glucose uptake and fatty acid oxidation is impaired in skeletal muscle from obese subjects. *Diabetes* **54**(11)**:** 3154-3160.

Campia U, Cardillo C, Panza J (2004). Ethnic differences in the vasoconstrictor activity of endogenous endothelin-1 in hypertensive patients. *Circulation* **109**(25)**:** 3191-3195.

Cardillo C, Campia U, Bryant MB, Panza JA (2002). Increased activity of endogenous endothelin in patients with type II diabetes mellitus. *Circulation* **106**(14)**:** 1783-1787.

Cardillo C, Campia U, Iantorno M, Panza JA (2004). Enhanced vascular activity of endogenous endothelin-1 in obese hypertensive patients. *Hypertension* **43**(1)**:** 36-40.

Cardillo C, Kilcoyne CM, Waclawiw M, Cannon RO, III, Panza JA (1999a). Role of Endothelin in the Increased Vascular Tone of Patients With Essential Hypertension. *Hypertension* **33**(2)**:** 753-758.

Cardillo C, Nambi SS, Kilcoyne CM, Choucair WK, Katz A, Quon MJ, Panza JA (1999b). Insulin stimulates both endothelin and nitric oxide activity in the human forearm. *Circulation* **100**(8)**:** 820-825.

Ceddia RB, Somwar R, Maida A, Fang X, Bikopoulos G, Sweeney G (2005). Globular adiponectin increases GLUT4 translocation and glucose uptake but reduces glycogen synthesis in rat skeletal muscle cells. *Diabetologia* **48**(1)**:** 132-139.

Charbonneau A, Marette A (2010). Inducible Nitric Oxide Synthase Induction Underlies Lipid-Induced Hepatic Insulin Resistance in Mice. *Diabetes* **59**(4)**:** 861-871.

Chen H, Montagnani M, Funahashi T, Shimomura I, Quon MJ (2003). Adiponectin stimulates production of nitric oxide in vascular endothelial cells. *J. Biol. Chem.* **278**(45)**:** 45021-45026.

Chen MB, McAinch AJ, Macaulay SL, Castelli LA, O'Brien PE, Dixon JB, Cameron-Smith D, Kemp BE, Steinberg GR (2005). Impaired activation of AMP-kinase and fatty acid oxidation by globular adiponectin in cultured human skeletal muscle of obese type 2 diabetics. *J. Clin. Endocrinol. Metab.* **90**(6)**:** 3665-3672.

Cheng KKY, Lam KSL, Wang Y, Huang Y, Carling D, Wu D, Wong C, Xu A (2007). Adiponectin-Induced Endothelial Nitric Oxide Synthase Activation and Nitric Oxide Production Are Mediated by APPL1 in Endothelial Cells. *Diabetes* **56**(5)**:** 1387-1394.

Clark M (2008). Impaired microvascular perfusion: a consequence of vascular dysfunction and a potential cause of insulin resistance in muscle. *American Journal of Physiology Endocrinology and Metabolism* **295**(4)**:** E732-750.

Clark MG, Colquhoun EQ, Rattigan S, Dora KA, Eldershaw TP, Hall JL, Ye J (1995). Vascular and endocrine control of muscle metabolism. *Am J Physiol Endocrinol Metab* **268**(5)**:** E797-E812.

Clark MG, Rattigan S, Clerk LH, Vincent MA, Clark ADH, Youd JM, Newman JMB (2000). Nutritive and non-nutritive blood flow: rest and exercise. *Acta Physiol. Scand.* **168**(4)**:** 519-530.

Clark MG, Wallis MG, Barrett EJ, Vincent MA, Richards SM, Clerk LH, Rattigan S (2003). Blood flow and muscle metabolism: a focus on insulin action. *Am J Physiol Endocrinol Metab* **284**(2)**:** E241-E258.

Clarke J, Benjamin N, Larkin S, Webb D, Davies G, Maseri A (1989). Endothelin is a potent long-lasting vasoconstrictor in men. *American Journal of Physiology Heart and Circulation Physiology* **257**(6 Pt 2)**:** H2033-2035.

Clerk L, Vincent M, Jahn L, Liu Z, Lindner J, Barrett E (2006). Obesity blunts insulin-mediated microvascular recruitment in human forearm muscle. *Diabetes* **55**(5)**:** 1436-1442.

Clerk LH, Rattigan S, Clark MG (2002). Lipid Infusion Impairs Physiologic Insulin-Mediated Capillary Recruitment and Muscle Glucose Uptake In Vivo. *Diabetes* **51**(4)**:** 1138-1145.

Clerk LH, Vincent MA, Barrett EJ, Lankford MF, Lindner JR (2007). Skeletal muscle capillary responses to insulin are abnormal in late-stage diabetes and are restored by angiogensin-converting enzyme inhibition. *American Journal of Physiology Endocrinology and Metabolism* **293**(6)**:** E1804-1809.

Coggins M, Lindner J, Rattigan S, Jahn L, Fasy E, Kaul S, Barrett E (2001). Physiologic Hyperinsulinemia Enhances Human Skeletal Muscle Perfusion by Capillary Recruitment. *Diabetes* **50**(12)**:** 2682-2690.

Colquhoun EQ, Hettiarachchi M, Ye J, Richter E, Hniat A, Rattigan S, Clark M (1988). Vasopressin and angiotensin II stimulate oxygen uptake in the perfused rat hindlimb. *Life Sci.* **43**(21)**:** 1747-1754.

Combs TP, Berg AH, Obici S, Scherer PE, Rossetti L (2001). Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. *J. Clin. Invest.* **108**(12)**:** 1875-1881.

Combs TP, Pajvani UB, Berg AH, Lin Y, Jelicks LA, Laplante M, Nawrocki AR, Rajala MW, Parlow AF, Cheeseboro L, Ding Y-Y, Russell RG, Lindemann D, Hartley A, Baker GRC, Obici S, Deshaies Y, Ludgate M, Rossetti L, Scherer PE (2004). A transgenic mouse with a deletion in the collagenous domain of adiponectin displays elevated circulating adiponectin and improved insulin sensitivity. *Endocrinology* **145**(1)**:** 367-383.

Combs TP, Wagner JA, Berger J, Doebber T, Wang W-J, Zhang BB, Tanen M, Berg AH, O'Rahilly S, Savage DB, Chatterjee K, Weiss S, Larson PJ, Gottesdiener KM, Gertz BJ, Charron MJ, Scherer PE, Moller DE (2002). Induction of adipocyte complement-related protein of 30 kilodaltons by PPARγ agonists: A potential mechanism of insulin sensitization. *Endocrinology* **143**(3)**:** 998-1007.

D'Orléans-Juste P, Claing A, Warner T, Yano M, Télémaque S (1993). Characterization of receptors for endothelins in the perfused arterial and venous mesenteric vasculatures of the rat. *Br. J. Pharmacol.* **110**(2)**:** 687-692.

da Silva AA, Kuo JJ, Tallam LS, Hall JE (2004). Role of Endothelin-1 in Blood Pressure Regulation in a Rat Model of Visceral Obesity and Hypertension. *Hypertension* **43**(2)**:** 383-387.

Dallaire P, Bellmann K, Laplante M, Gelinas S, Centeno-Baez C, Penfornis P, Peyot M-L, Latour MG, Lamontagne J, Trujillo ME, Scherer PE, Prentki M, Deshaies Y, Marette A (2008). Obese Mice Lacking Inducible Nitric Oxide Synthase Are Sensitized to the Metabolic Actions of Peroxisome Proliferator-Activated Receptor-gamma Agonism. *Diabetes* **57**(8)**:** 1999-2011.

Dawson D, Vincent MA, Barrett EJ, Kaul S, Clark A, Leong-Poi H, Lindner JR (2002). Vascular recruitment in skeletal muscle during exercise and hyperinsulinemia assessed by contrast ultrasound. *Am J Physiol Endocrinol Metab* **282**(3)**:** E714-720.

de Jongh R, Serne EH, IJzerman RG, de Vries G, Stehouwer CD. (2004). Impaired microvascular function in obesity: implications for obesity-associated microangiopathy, hypertension, and insulin resistance. *Circulation* **109**(21)**:** 2529-2535.

DeFronzo R, Gunnarsson R, Björkman O, Olsson M, Wahren J (1985). Effects of insulin on peripheral and splanchnic glucose metabolism in noninsulin-dependent (type II) diabetes mellitus. *J. Clin. Invest.* **76**(1)**:** 149-155.

Donovan C, Hamilton-Wessler M, Halter J, Bergman R (1994). Primacy of liver glucosensors in the sympathetic response to progressive hypoglycemia. *Proc. Natl. Acad. Sci. U. S. A.* **91**(7)**:** 2863-2867.

Dora KA, Richards SM, Rattigan S, Colquhoun EQ, Clark MG (1992). Serotonin and norepinephrine vasoconstriction in rat hindlimb have different oxygen requirements. *Am J Physiol Heart Circ Physiol* **262**(3)**:** H698-H703.

Dreja K, Voldstedlund M, Vinten J, Tranum-Jensen J, Hellstrand P, Sward K (2002). Cholesterol Depletion Disrupts Caveolae and Differentially Impairs Agonist-Induced Arterial Contraction. *Arterioscler. Thromb. Vasc. Biol.* **22**(8)**:** 1267-1272.

Dunstan D, Zimmet P, Welborn T, De Courten M, Cameron A, Sicree R, Dwyer T, Colagiuri S, Jolley D, Knuiman M, Atkins R, Shaw J (2002). The rising prevalence of diabetes and impaired glucose tolerance: the Australian Diabetes, Obesity and Lifestyle Study. *Diabetes Care* **25**(5)**:** May.

Dyck DJ, Heigenhauser GJF, Bruce CR (2006). The role of adipokines as regulators of skeletal muscle fatty acid metabolism and insulin sensitivity. *Acta Physiologica* **186**(1)**:** 5-16.

Elgebaly M, Kelly A, Harris A, Elewa H, Portik-Dobos V, Ketsawatsomkron P, Marrero M, Ergul A (2008). Impaired insulin-mediated vasorelaxation in a nonobese model of type 2 diabetes: role of endothelin-1. *Can J Physiol Pharmacol.* **86**(6)**:** 358-364.

Emmerson B, Gordon R, Cross M, Thomson D (1987). Plasma oxipurinol concentrations during allopurinol therapy. *Rheumatology (Oxford).* **26**(6)**:** 445-449.

English JM, Cobb MH (2002). Pharmacological inhibitors of MAPK pathways. *Trends Pharmacol. Sci.* **23**(1)**:** 40-45.

English PJ, Coughlin SR, Hayden K, Malik IA, Wilding JPH (2003). Plasma Adiponectin Increases Postprandially in Obese, but not in Lean, Subjects. *Obesity* **11**(7)**:** 839-844.

Eringa EC, Bakker W, Smulders YM, SernÃ© EH, Yudkin JS, Stehouwer CDA (2007). Regulation of Vascular Function and Insulin Sensitivity by Adipose Tissue: Focus on Perivascular Adipose Tissue. *Microcirculation* **14**(4)**:** 389 - 402.

Eringa EC, Stehouwer CDA, Merlijn T, Westerhof N, Sipkema P (2002). Physiological concentrations of insulin induce endothelin-mediated vasoconstriction during inhibition of NOS or PI3-kinase in skeletal muscle arterioles. *Cardiovasc. Res.* **56**(3)**:** 464-471.

Ferri C, Bellini C, Desideri G, De Mattia G, Santucci A (1996). Endogenous insulin modulates circulating endothelin-1 concentrations in humans. *Diabetes Care* **19**(5)**:** 504-506.

Ferri C, Bellini C, Desideri G, Francesco LD, Baldoncini R, Santucci A, Mattia GD (1995). Plasma endothelin-1 levels in obese hypertensive and normotensive men. *Diabetes* **44**(4)**:** 431-436.

Ferro CJ, Haynes WG, Hand MF, Webb DJ (2002). Forearm vasoconstriction to endothelin-1 is impaired, but constriction to sarafotoxin 6c and vasodilatation to BQ-123 unaltered, in patients with essential hypertension. *Clin. Sci.* **103 Suppl 48:** 53S-58S.

Fesus G, Dubrovska G, Gorzelniak K, Kluge R, Huang Y, Luft FC, Gollasch M (2007). Adiponectin is a novel humoral vasodilator. *Cardiovasc. Res.* **75**(4)**:** 719-727.

Frank HJ, Levin ER, Hu RM, Pedram A (1993). Insulin stimulates endothelin binding and action on cultured vascular smooth muscle cells. *Endocrinology* **133**(3)**:** 1092-1097.

Fruebis J, Tsao T, Javorschi S, Ebbets-Reed D, Erickson M, Yen F, Bihain B, Lodish H (2001). Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc. Natl. Acad. Sci. U. S. A.* **98**(4)**:** 2005-2010.

Fujimoto M, Shimizu N, Kunii K, Martyn JAJ, Ueki K, Kaneki M (2005). A Role for iNOS in Fasting Hyperglycemia and Impaired Insulin Signaling in the Liver of Obese Diabetic Mice. *Diabetes* **54**(5)**:** 1340-1348.

Fujioka D, Kawabata K-i, Saito Y, Kobayashi T, Nakamura T, Kodama Y, Takano H, Obata J-e, Kitta Y, Umetani K, Kugiyama K (2006). Role of adiponectin receptors in endothelin-induced cellular hypertrophy in cultured cardiomyocytes and their expression in infarcted heart. *Am J Physiol Heart Circ Physiol* **290**(6)**:** H2409-H2416.

Furukawa K, Hori M, Ouchi N, Kihara S, Funahashi T, Matsuzawa Y, Miyazaki A, Nakayama H, Horiuchi S (2004). Adiponectin down-regulates acyl-coenzyme A:cholesterol acyltransferase-1 in cultured human monocyte-derived macrophages. *Biochem. Biophys. Res. Commun.* **317**(3)**:** 831-836.

Furutani H, Zhang X, Iwamuro Y, Lee K, Okamoto Y, Takikawa O, Fukao M, Masaki T, Miwa S (2002). Ca^{2+} entry channels involved in contractions of rat aorta induced by endothelin-1, noradrenaline, and vasopressin. *J. Cardiovasc. Pharmacol.* **40**(2)**:** 265-276.

Gaberc-Porekar V, Menart V (2005). Potential for Using Histidine Tags in Purification of Proteins at Large Scale. *Chemical Engineering & Technology* **28**(11)**:** 1306-1314.

Gaillard P (1970). Induction of Bone Formation in Explanted Bone Rudiments by Calcitone and Imidazole. *Calc. Tiss. Res* **4**(supplement)**:** 86-87.

Galluccio M, Brizio C, Torchetti EM, Ferranti P, Gianazza E, Indiveri C, Barile M (2007). Over-expression in Escherichia coli, purification and characterization of isoform 2 of human FAD synthetase. *Protein Expr. Purif.* **52**(1)**:** 175-181.

Garvey EP, Oplinger JA, Furfine ES, Kiff RJ, Laszlo F, Whittle BJR, Knowles RG (1997). 1400W Is a Slow, Tight Binding, and Highly Selective Inhibitor of Inducible Nitric-oxide Synthase in Vitro and in Vivo. *J. Biol. Chem.* **272**(8)**:** 4959-4963.

Goldstein BJ, Scalia R (2004). Adiponectin: A Novel Adipokine Linking Adipocytes and Vascular Function. *J. Clin. Endocrinol. Metab.* **89**(6)**:** 2563-2568.

Gonon AT, Widegren U, Bulhak A, Salehzadeh F, Persson J, Sjoquist P-O, Pernow J (2008). Adiponectin protects against myocardial ischaemia-reperfusion injury via AMP-activated protein kinase, Akt, and nitric oxide. *Cardiovasc. Res.* **78**(1)**:** 116-122.

Greene EC (1968). *Anatomy of the rat*. edn. Hafner Publishing Co.: U.S.A.

Grodberg J, Dunn J (1988). ompT encodes the Escherichia coli outer membrane protease that cleaves T7 RNA polymerase during purification. *J. Bacteriol.* **170**(3)**:** 1245-1253.

Guang-Yao S, Yu G, Yu-Wei D, Li-Li P, Yu Z, Ji-Ming Y (2006). High-fat feeding reduces endothelium-dependent vasodilation in rats: differential mechanisms for saturated and unsaturated fatty acids? *Clin. Exp. Pharmacol. Physiol.* **33**(8)**:** 708-713.

Gunnett CA, Lund DD, Chu Y, Brooks RM, II, Faraci FM, Heistad DD (2001). NO-Dependent Vasorelaxation Is Impaired After Gene Transfer of Inducible NO-Synthase. *Arterioscler. Thromb. Vasc. Biol.* **21**(8)**:** 1281-1287.

Halberg N, Schraw T, Wang Z, Kim J, Yi J, Hamilton M, Luby-Phelps K, Scherer P (2009). Systemic fate of the adipocyte-derived factor adiponectin. *Diabetes* **58**(9)**:** 1961-1970.

Halperin F, Beckman J, Patti M, Trujillo M, Garvin M, Creager M, Scherer P, Goldfine A (2005). The role of total and high-molecular-weight complex of adiponectin in vascular function in offspring whose parents both had type 2 diabetes. *Diabetologia* **48**(10)**:** 2147-2154.

Halseth AE, Bracy DP, Wasserman DH (1998). Limitations to exercise- and maximal insulin-stimulated muscle glucose uptake. *J. Appl. Physiol.* **85**(6)**:** 2305-2313.

Halseth AE, Bracy DP, Wasserman DH (2000). Limitations to basal and insulin-stimulated skeletal muscle glucose uptake in the high-fat-fed rat. *Am J Physiol Endocrinol Metab* **279**(5)**:** E1064-1071.

Han S-Z, Ouchi Y, Karaki H, Orimo H (1995). Inhibitory Effects of Insulin on Cytosolic Ca2+ Level and Contraction in the Rat Aorta : Endothelium-Dependent and -Independent Mechanisms. *Circ. Res.* **77**(4)**:** 673-678.

Harrison DK, Birkenhake S, Knauf SK, Kessler M (1990). Local oxygen supply and blood flow regulation in contracting muscle in dogs and rabbits. *The Journal of Physiology* **422**(1)**:** 227-243.

Hattori Y, Nakano Y, Hattori S, Tomizawa A, Inukai K, Kasai K (2008). High molecular weight adiponectin activates AMPK and suppresses cytokine-induced NF-κB activation in vascular endothelial cells. *FEBS Lett.* **582**(12)**:** 1719-1724.

Hattori Y, Suzuki M, Hattori S, Kasai K (2003). Globular adiponectin upregulates nitric oxide production in vascular endothelial cells. *Diabetologia* **46**(11)**:** 1543-1549.

Henriksen EJ, Teachey MK, Lindborg KA, Diehl CJ, Beneze AN (2008). The high-fat-fed lean Zucker rat: a spontaneous isocaloric model of fat-induced insulin resistance associated with muscle GSK-3 overactivity. *Am J Physiol Regul Integr Comp Physiol* **294**(6)**:** R1813-1821.

Hirata Y, Yoshimi H, Takaichi S, Yanagisawa M, Masaki T (1988). Binding and receptor down-regulation of a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. *FEBS Lett.* **239**(1)**:** 13-17.

Hocher B, Schwarz A, Slowinski T, Bachmann S, Pfeilschifter J, Neumayer HH, Bauer C (2004). In-vivo interaction of nitric oxide and endothelin. *J. Hypertens.* **22**(1)**:** 111-119.

Hopfner RL, Hasnadka RV, Wilson TW, McNeill JR, Gopalakrishnan V (1998). Insulin increases endothelin-1-evoked intracellular free calcium responses by increased ET(A) receptor expression in rat aortic smooth muscle cells. *Diabetes* **47**(6)**:** 937-944.

Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y (2000). Plasma Concentrations of a Novel, Adipose-Specific Protein, Adiponectin, in Type 2 Diabetic Patients. *Arterioscler. Thromb. Vasc. Biol.* **20**(6)**:** 1595-1599.

Hu E, Liang P, Spiegelman BM (1996). AdipoQ Is a Novel Adipose-specific Gene Dysregulated in Obesity. *J. Biol. Chem.* **271**(18)**:** 10697-10703.

Hug C, Wang J, Ahmad N, Bogan J, Tsao T, Lodish H (2004). T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. *Proc. Natl. Acad. Sci. U. S. A.* **101**(28)**:** 10308-10313.

Ignarro L (1990). Nitric oxide. A novel signal transduction mechanism for transcellular communication. *Hypertension* **16**(5)**:** 477-483.

Ishibashi K-i, Imamura T, Sharma PM, Huang J, Ugi S, Olefsky JM (2001). Chronic endothelin-1 treatment leads to heterologous desensitization of insulin signaling in 3T3-L1 adipocytes. *J. Clin. Invest.* **107**(9)**:** 1193-1202.

Iwashima Y, Katsuya T, Ishikawa K, Ouchi N, Ohishi M, Sugimoto K, Fu Y, Motone M, Yamamoto K, Matsuo A, Ohashi K, Kihara S, Funahashi T, Rakugi H, Matsuzawa Y, Ogihara T (2004). Hypoadiponectinemia is an independent risk factor for hypertension. *Hypertension* **43**(6)**:** 1318-1323.

Iwashina M, Shichiri M, Marumo F, Hirata Y (1998). Transfection of Inducible Nitric Oxide Synthase Gene Causes Apoptosis in Vascular Smooth Muscle Cells. *Circulation* **98**(12)**:** 1212-1218.

James DE, Jenkins AB, Kraegen EW (1985). Heterogeneity of insulin action in individual muscles in vivo: euglycemic clamp studies in rats. *Am J Physiol Endocrinol Metab* **248**(5)**:** E567-574.

Jarasch E, Bruder G, Heid H (1986). Significance of xanthine oxidase in capillary endothelial cells. *Acta Physiologica Scand* **548:** 39-46.

Juan C-C, Shen Y-W, Chien Y, Lin Y-J, Chang S-F, Ho L-T (2004). Insulin infusion induces endothelin-1-dependent hypertension in rats. *Am J Physiol Endocrinol Metab* **287**(5)**:** E948-954.

Juan C, Fang V, Hsu Y, Huang Y, Hsia D, Yu P, Kwok C, Ho L (1998). Overexpression of vascular endothelin-1 and endothelin-A receptors in a fructose-induced hypertensive rat model. *J. Hypertens.* **16**(12 Pt 1)**:** 1775-1782.

Kadowaki T, Yamauchi T (2005). Adiponectin and Adiponectin Receptors. *Endocr. Rev.* **26**(3)**:** 439-451.

Kadowaki T, Yamauchi T, Kubota N (2008). The physiological and pathophysiological role of adiponectin and adiponectin receptors in the peripheral tissues and CNS. *FEBS Lett.* **582**(1)**:** 74-80.

Kapur S, Bédard S, Marcotte B, Côté C, Marette A (1997). Expression of nitric oxide synthase in skeletal muscle: a novel role for nitric oxide as a modulator of insulin action. *Diabetes* **46**(11)**:** 691-1700.

Kapur S, Marcotte B, Marette A (1999). Mechanism of adipose tissue iNOS induction in endotoxemia. *Am J Physiol Endocrinol Metab* **276**(4)**:** E635-641.

Kashyap S, Roman L, Mandarino L, DeFronzo R, Bajaj M (2010). Hypoadiponectinemia is closely associated with impaired nitric oxide synthase activity in skeletal muscle of type 2 diabetic subjects. *Metabolic Syndrome and Related Disorders* **8**(5)**:** 459-463.

Katakam PVG, Pollock JS, Pollock DM, Ujhelyi MR, Miller AW (2001). Enhanced endothelin-1 response and receptor expression in small mesenteric arteries of insulin-resistant rats. *Am J Physiol Heart Circ Physiol* **280**(2)**:** H522-527.

Katakam PVG, Snipes JA, Tulbert CD, Mayanagi K, Miller AW, Busija DW (2006). Impaired endothelin-induced vasoconstriction in coronary arteries of Zucker obese rats is associated with uncoupling of [Ca2+]i signaling. *Am J Physiol Regul Integr Comp Physiol* **290**(1)**:** R145-153.

Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G (2003). Adiponectin expression from human adipose tissue: Relation to obesity, insulin resistance, and tumor necrosis factor-α expression. *Diabetes* **52**(7)**:** 1779-1785.

Keske M, Clerk L, Price W, Jahn L, Barrett E (2009). Obesity blunts microvascular recruitment in human forearm muscle after a mixed meal. *Diabetes Care* **32**(9)**:** 1672-1677.

Kessler P, Bauersachs J, Busse R, Schini-Kerth VB (1997). Inhibition of Inducible Nitric Oxide Synthase Restores Endothelium-Dependent Relaxations in Proinflammatory Mediator-Induced Blood Vessels. *Arterioscler. Thromb. Vasc. Biol.* **17**(9)**:** 1746-1755.

Kim F, Gallis B, Corson MA (2001). TNF-α inhibits flow and insulin signaling leading to NO production in aortic endothelial cells. *American Journal of Physiology Cell Physiology* **280**(5)**:** C1057-1065.

Kim F, Pham M, Maloney E, Rizzo NO, Morton GJ, Wisse BE, Kirk EA, Chait A, Schwartz MW (2008). Vascular Inflammation, Insulin Resistance, and Reduced Nitric Oxide Production Precede the Onset of Peripheral Insulin Resistance. *Arterioscler. Thromb. Vasc. Biol.* **28**(11)**:** 1982-1988.

Kim F, Tysseling KA, Rice J, Pham M, Haji L, Gallis BM, Baas AS, Paramsothy P, Giachelli CM, Corson MA, Raines EW (2005). Free Fatty Acid Impairment of Nitric Oxide Production in Endothelial Cells Is Mediated by IKK{beta}. *Arterioscler. Thromb. Vasc. Biol.* **25**(5)**:** 989-994.

Kim J-a, Montagnani M, Koh KK, Quon MJ (2006). Reciprocal Relationships Between Insulin Resistance and Endothelial Dysfunction: Molecular and Pathophysiological Mechanisms. *Circulation* **113**(15)**:** 1888-1904.

Kim J-Y, van de Wall E, Laplante M, Azzara A, Trujillo ME, Hofmann SM, Schraw T, Durand JL, Li H, Li G, Jelicks LA, Mehler MF, Hui DY, Deshaies Y, Shulman GI, Schwartz GJ, Scherer PE (2007). Obesity-associated improvements in metabolic profile through expansion of adipose tissue. *J. Clin. Invest.* **117**(9)**:** 2621-2637.

Kingwell B, Formosa M, Muhlmann M, Bradley S, McConell G (2002). Nitric oxide synthase inhibition reduces glucose uptake during exercise in individuals with type 2 diabetes more than in control subjects. *Diabetes* **51**(8)**:** 2572-2580.

Kobayashi H, Ouchi N, Kihara S, Walsh K, Kumada M, Abe Y, Funahashi T, Matsuzawa Y (2004). Selective Suppression of Endothelial Cell Apoptosis by the High Molecular Weight Form of Adiponectin. *Circ. Res.* **94**(4)**:** e27-31.

Kolka CM (2006). Glucose Uptake in Skeletal Muscle. Doctor of Philosophy, University of Tasmania, Hobart.

Kolka CM, Rattigan S, Richards S, Clark MG (2005). Metabolic and vascular actions of endothelin-1 are inhibited by insulin-mediated vasodilation in perfused rat hindlimb muscle. *Br. J. Pharmacol.* **145**(7)**:** 992-1000.

Kondo M, Shibata R, Miura R, Shimano M, Kondo K, Li P, Ohashi T, Kihara S, Maeda N, Walsh K, Ouchi N, Murohara T (2009). Caloric Restriction Stimulates Revascularization in Response to Ischemia via Adiponectin-mediated Activation of Endothelial Nitric-oxide Synthase. *J. Biol. Chem.* **284**(3)**:** 1718-1724.

Kou G, Shi S, Wang H, Tan M, Xue J, Zhang D, Hou S, Qian W, Wang S, Dai J (2007). Preparation and characterization of recombinant protein ScFv(CD11c)-TRP2 for tumor therapy from inclusion bodies in Escherichia coli. *Protein Expr. Purif.* **52**(1)**:** 131-138.

Kraegen EW, James DE, Jenkins AB, Chisholm DJ (1985). Dose-response curves for in vivo insulin sensitivity in individual tissues in rats. *Am J Physiol Endocrinol Metab* **248**(3)**:** E353-362.

Kriketos A, Gan SK, Poynten AM, Furler SM, Chisholm DJ, Campbell LV. (2004). Exercise increases adiponectin levels and insulin sensitivity in humans. *Diabetes Care* **27**(2)**:** 629-630.

Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, Eto K, Yamashita T, Kamon J, Satoh H, Yano W, Froguel P, Nagai R, Kimura S, Kadowaki T, Noda T (2002). Disruption of Adiponectin Causes Insulin Resistance and Neointimal Formation. *J. Biol. Chem.* **277**(29)**:** 25863-25866.

Kubota N, Yano W, Kubota T, Yamauchi T, Itoh S, Kumagai H, Kozono H, Takamoto I, Okamoto S, Shiuchi T, Suzuki R, Satoh H, Tsuchida A, Moroi M, Sugi K, Noda T, Ebinuma H, Ueta Y, Kondo T, Araki E, Ezaki O, Nagai R, Tobe K, Terauchi Y, Ueki K, Minokoshi Y, Kadowaki T (2007). Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. *Cell Metabolism* **6**(1)**:** 55-68.

Kusminski C, McTernan P, Schraw T, Kos K, Oâ€™Hare J, Ahima R, Kumar S, Scherer P (2007). Adiponectin complexes in human cerebrospinal fluid: distinct complex distribution from serum. *Diabetologia* **50**(3)**:** 634-642.

Laakso M, Edelman S, Brechtel G, Baron A (1992). Impaired insulin-mediated skeletal muscle blood flow in patients with NIDDM. *Diabetes* **41**(9)**:** 1076-1083.

Laakso M, Edelman SV, Brechtel G, Baron AD. (1990). Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man. A novel mechanism for insulin resistance. *J. Clin. Invest.* **85:** 1844-1852.

Laight DW, Martin J. Carrier, Erik E. Änggård, (1999). Endothelial cell dysfunction and the pathogenesis of diabetic macroangiopathy. *Diabetes. Metab. Res. Rev.* **15**(4)**:** 274-282.

Lara-Castro C, Luo N, Wallace P, Klein R, Garvey W (2006). Adiponectin multimeric complexes and the metabolic syndrome trait cluster. *Diabetes* **55**(1)**:** 249-259.

Lau DCW, Dhillon B, Yan H, Szmitko PE, Verma S (2005). Adipokines: molecular links between obesity and atheroslcerosis. *Am J Physiol Heart Circ Physiol* **288**(5)**:** H2031-2041.

Lesniewski LA, Donato AJ, Behnke BJ, Woodman CR, Laughlin MH, Ray CA, Delp MD (2008). Decreased NO signaling leads to enhanced vasoconstrictor responsiveness in skeletal muscle arterioles of the ZDF rat prior to overt diabetes and hypertension. *Am J Physiol Heart Circ Physiol* **294**(4)**:** H1840-1850.

Levin ER (1995). Endothelins. *N. Engl. J. Med.* **333**(6)**:** 356-363.

Lin HV, Kim J-Y, Pocai A, Rossetti L, Shapiro L, Scherer PE, Accili D (2007). Adiponectin Resistance Exacerbates Insulin Resistance in Insulin Receptor Transgenic/Knockout Mice. *Diabetes* **56**(8)**:** 1969-1976.

Ling P, Bistrian B, Mendez B, Istfan N (1994). Effects of systemic infusions of endotoxin, tumor necrosis factor, and interleukin-1 on glucose metabolism in the rat: relationship to endogenous glucose production and peripheral tissue glucose uptake. *Metabolism.* **43**(3)**:** 279-284.

Liu Y, Chewchuk S, Lavigne C, Brule S, Pilon G, Houde V, Xu A, Marette A, Sweeney G (2009a). Functional significance of skeletal muscle adiponectin production, changes in animal models of obesity and diabetes, and regulation by rosiglitazone treatment. *Am J Physiol Endocrinol Metab* **297**(3)**:** E657-664.

Liu Z, Liu J, Jahn LA, Fowler DE, Barrett EJ (2009b). Infusing Lipid Raises Plasma Free Fatty Acids and Induces Insulin Resistance in Muscle Microvasculature. *J. Clin. Endocrinol. Metab.* **94**(9)**:** 3543-3549.

Lloréns S, Miranda FJ, Alabadí JA, Marrachelli VG, Alborch E (2004). Different role of endothelin ETA and ETB receptors and endothelial modulators in diabetes-induced hyperreactivity of the rabbit carotid artery to endothelin-1. *Eur. J. Pharmacol.* **486**(1)**:** 43-51.

Lloyd CE, Becker D, Ellis D, Orchard TJ (1996). Incidence of Complications in Insulin-dependent Diabetes Mellitus: A Survival Analysis. *Am. J. Epidemiol.* **143**(5)**:** 431-441.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**(1)**:** 265-275.

Lteif AA, Fulford AD, Considine RV, Gelfand I, Baron AD, Mather KJ (2008). Hyperinsulinemia fails to augment ET-1 action in the skeletal muscle vascular bed in vivo in humans. *Am J Physiol Endocrinol Metab* **295**(6)**:** E1510-1517.

Ma K, Cabrero A, Saha PK, Kojima H, Li L, Chang BH-J, Paul A, Chan L (2002). Increased Beta-Oxidation but No Insulin Resistance or Glucose Intolerance in Mice Lacking Adiponectin. *J. Biol. Chem.* **277**(38)**:** 34658-34661.

Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, Matsubara K (1996). cDNA Cloning and Expression of a Novel Adipose Specific Collagen-like Factor, apM1 (dioseost Abundant Gene Transcript 1). *Biochem. Biophys. Res. Commun.* **221**(2)**:** 286-289.

Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y, Komuro R, Ouchi N, Kihara S, Tochino Y, Okutomi K, Horie M, Takeda S, Aoyama T, Funahashi T, Matsuzawa Y (2002a). Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat. Med.* **8**(7)**:** 731-737.

Maeda S, Miyauchi T, Iemitsu M, Tanabe T, Irukayama-Tomobe Y, Goto K, Yamaguchi I, Matsuda M (2002b). Involvement of Endogenous Endothelin-1 in Exercise-Induced Redistribution of Tissue Blood Flow: An Endothelin Receptor Antagonist Reduces the Redistribution. *Circulation* **106**(17)**:** 2188-2193.

Maeda S, Miyauchi T, Sakane M, Saito M, Maki S, Goto K, Matsuda M (1997). Does endothelin-1 participate in the exercise-induced changes of blood flow distribution of muscles in humans? *J. Appl. Physiol.* **82**(4)**:** 1107-1111.

Maguire J, Davenport A (1995). ETA receptor-mediated constrictor responses to endothelin peptides in human blood vessels in vitro. *Br. J. Pharmacol.* **115**(1)**:** 191-197.

Mather KJ, Lteif A, Steinberg HO, Baron AD (2004). Interactions between endothelin and nitric oxide in the regulation of vascular tone in obesity and diabetes. *Diabetes* **53**(8)**:** 2060-2066.

Mather KJ, Mirzamohammadi B, Lteif A, Steinberg HO, Baron AD (2002). Endothelin contributes to basal vascular tone and endothelial dysfunction in human obesity and type 2 diabetes. *Diabetes* **51**(12)**:** 3517-3523.

Matsuura F, Oku H, Koseki M, Sandoval JC, Yuasa-Kawase M, Tsubakio-Yamamoto K, Masuda D, Maeda N, Tsujii K-i, Ishigami M, Nishida M, Hirano K-i, Kihara S, Hori M, Shimomura I, Yamashita S (2007). Adiponectin accelerates reverse cholesterol transport by increasing high density lipoprotein assembly in the liver. *Biochem. Biophys. Res. Commun.* **358**(4)**:** 1091-1095.

McAinch AJ, Steinberg GR, Mollica J, O'Brien PE, Dixon JB, Macaulay SL, Kemp BE, Cameron-Smith D (2006). Differential Regulation of Adiponectin Receptor Gene Expression by Adiponectin and Leptin in Myotubes Derived from Obese and Diabetic Individuals. *Obesity* **14**(11)**:** 1898-1904.

McAuley D, McGurk C, Nugent A, Hanratty C, Hayes J, Johnston G (2000a). Vasoconstriction to endothelin-1 is blunted in non-insulin-dependent diabetes: a dose-response study. *J. Cardiovasc. Pharmacol.* **36**(2)**:** 203-208.

McAuley DF, Nugent AG, McGurk C, Maguire S, Hayes JR, Johnston GD (2000b). Vasoconstriction to endogenous endothelin-1 is impaired in patients with type II diabetes mellitus. *Clin. Sci.* **99**(3)**:** 175-179.

Miller AW, Tulbert C, Puskar M, Busija DW (2002). Enhanced Endothelin Activity Prevents Vasodilation to Insulin in Insulin Resistance. *Hypertension* **40**(1)**:** 78-82.

Mullen KL, Pritchard J, Ritchie I, Snook LA, Chabowski A, Bonen A, Wright D, Dyck DJ (2009). Adiponectin resistance precedes the accumulation of skeletal muscle lipids and insulin resistance in high-fat-fed rats. *Am J Physiol Regul Integr Comp Physiol* **296**(2)**:** R243-251.

Mullen KL, Smith AC, Junkin KA, Dyck DJ (2007). Globular adiponectin resistance develops independently of impaired insulin-stimulated glucose transport in soleus muscle from high-fat-fed rats. *American Journal of Physiology Endocrinology and Metabolism* **293**(1)**:** E83-90.

Muniyappa R, Quon M (2007). Insulin action and insulin resistance in vascular endothelium. *Current opinion in clinical nutrition and metabolic care* **10**(4)**:** 523-530.

Nakano Y, Tobe T, Choi-Miura N-H, Mazda T, Tomita M (1996). Isolation and Characterization of GBP28, a Novel Gelatin-Binding Protein Purified from Human Plasma. *J. Biochem. (Tokyo).* **120**(4)**:** 803-812.

Nathan C (1997). Inducible nitric oxide synthase: what difference does it make? *The Journal of Clinical Investigation* **100**(10)**:** 2417-2423.

Nawrocki AR, Rajala MW, Tomas E, Pajvani UB, Saha AK, Trumbauer ME, Pang Z, Chen AS, Ruderman NB, Chen H, Rossetti L, Scherer PE (2006). Mice Lacking Adiponectin Show Decreased Hepatic Insulin Sensitivity and Reduced Responsiveness to Peroxisome Proliferator-activated Receptor γ Agonists. *J. Biol. Chem.* **281**(5)**:** 2654-2660.

Newman JM, Dora KA, Rattigan S, Edwards SJ, Colquhoun EQ, Clark MG (1996). Norepinephrine and serotonin vasoconstriction in rat hindlimb control different vascular flow routes. *Am J Physiol Endocrinol Metab* **270**(4)**:** E689-699.

Nolte L, Hansen PA, Chen MM, Schluter JM, Gulve EA, Holloszy JO. (1998). Short-term exposure to tumor necrosis factor-alpha does not affect insulin-stimulated glucose uptake in skeletal muscle. *Diabetes* **47**(5)**:** 721-726.

Noriaki K, Sumio A, Noboru Y, Kazuaki N, Yoshihisa Y (2009). Anti-inflammatory activity of a globular adiponectin function on RAW 264 cells stimulated by lipopolysaccharide from *Aggregatibacter actinomycetemcomitans*. *FEMS Immunol. Med. Microbiol.* **56**(3)**:** 241-247.

Noronha BT, Li J-M, Wheatcroft SB, Shah AM, Kearney MT (2005). Inducible Nitric Oxide Synthase Has Divergent Effects on Vascular and Metabolic Function in Obesity. *Diabetes* **54**(4)**:** 1082-1089.

Nugent A, McGurk C, Hayes J, Johnston G (1996). Impaired vasoconstriction to endothelin 1 in patients with NIDDM. *Diabetes* **45**(1)**:** 105-107.

Ohashi K, Kihara S, Ouchi N, Kumada M, Fujita K, Hiuge A, Hibuse T, Ryo M, Nishizawa H, Maeda N, Maeda K, Shibata R, Walsh K, Funahashi T, Shimomura I (2006). Adiponectin Replenishment Ameliorates Obesity-Related Hypertension. *Hypertension* **47**(6)**:** 1108-1116.

Ohashi K, Ouchi N, Sato K, Higuchi A, Ishikawa T-o, Herschman HR, Kihara S, Walsh K (2009). Adiponectin Promotes Revascularization of Ischemic Muscle through a Cyclooxygenase 2-Dependent Mechanism. *Mol. Cell. Biol.* **29**(13)**:** 3487-3499.

Okamoto M, Ohara-Imaizumi M, Kubota N, Hashimoto S, Eto K, Kanno T, Kubota T, Wakui M, Nagai R, Noda M, Nagamatsu S, Kadowaki T (2008). Adiponectin induces insulin secretion in vitro and in vivo at a low glucose concentration. *Diabetologia* **51**(5)**:** 827-835.

Okamoto Y, Kihara S, Ouchi N, Nishida M, Arita Y, Kumada M, Ohashi K, Sakai N, Shimomura I, Kobayashi H, Terasaka N, Inaba T, Funahashi T, Matsuzawa Y (2002). Adiponectin Reduces Atherosclerosis in Apolipoprotein E-Deficient Mice. *Circulation* **106**(22)**:** 2767-2770.

Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y (1999). Novel modulator for endothelial adhesion molecules : Adipocyte-derived plasma protein adiponectin. *Circulation* **100**(25)**:** 2473-2476.

Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T, Matsuzawa Y (2001). Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* **103**(8)**:** 1057-1063.

Ouchi N, Kobayashi H, Kihara S, Kumada M, Sato K, Inoue T, Funahashi T, Walsh K (2004). Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. *J. Biol. Chem.* **279**(2)**:** 1304-1309.

Ouchi N, Ohishi M, Kihara S, Funahashi T, Nakamura T, Nagaretani H, Kumada M, Ohashi K, Okamoto Y, Nishizawa H, Kishida K, Maeda N, Nagasawa A, Kobayashi H, Hiraoka H, Komai N, Kaibe M, Rakugi H, Ogihara T, Matsuzawa Y (2003). Association of hypoadiponectinemia with impaired vasoreactivity. *Hypertension* **42**(3)**:** 231-234.

Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, Engel J, Brownlee M, Scherer PE (2003). Structure-Function Studies of the Adipocyte-secreted Hormone Acrp30/Adiponectin: Implications for metabolic regulation and bioactivity. *J. Biol. Chem.* **278**(11)**:** 9073-9085.

Pajvani UB, Hawkins M, Combs TP, Rajala MW, Doebber T, Berger JP, Wagner JA, Wu M, Knopps A, Xiang AH, Utzschneider KM, Kahn SE, Olefsky JM, Buchanan TA, Scherer PE (2004). Complex Distribution, Not Absolute Amount of Adiponectin,

Correlates with Thiazolidinedione-mediated Improvement in Insulin Sensitivity. *J. Biol. Chem.* **279**(13)**:** 12152-12162.

Perreault M, Marette A (2001). Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. *Nat. Med.* **7**(10)**:** 1138-1143.

Pilon G, Dallaire P, Marette A (2004). Inhibition of Inducible Nitric-oxide Synthase by Activators of AMP-activated Protein Kinase. *J. Biol. Chem.* **279**(20)**:** 20767-20774.

Plusczyk T, Bersal B, Menger MD, Feifel G (2001). Differential Effects of ET-1, ET-2, and ET-3 on Pancreatic Microcirculation, Tissue Integrity, and Inflammation. *Dig. Dis. Sci.* **46**(6)**:** 1343-1351.

Pollock DM, Keith TL, Highsmith RF (1995). Endothelin receptors and calcium signaling. *FASEB J.* **9**(12)**:** 1196-1204.

Potenza MA, Marasciulo FL, Tarquinio M, Quon MJ, Montagnani M (2006). Treatment of Spontaneously Hypertensive Rats With Rosiglitazone and/or Enalapril Restores Balance Between Vasodilator and Vasoconstrictor Actions of Insulin With Simultaneous Improvement in Hypertension and Insulin Resistance. *Diabetes* **55**(12)**:** 3594-3603.

Potter R, Groom A (1983). Capillary diameter and geometry in cardiac and skeletal muscle studied by means of corrosion casts. *Microvasc. Res.* **25**(1)**:** 68-84.

Qi Y, Takahashi N, Hileman SM, Patel HR, Berg AH, Pajvani UB, Scherer PE, Ahima RS (2004). Adiponectin acts in the brain to decrease body weight. *Nat. Med.* **10**(5)**:** 524-529.

Rattigan S, Appleby GJ, Miller KA, Steen JT, Dora KA, Colquhoun EQ, Clark MG (1997a). Serotonin inhibition of 1-methylxanthine metabolism parallels its vasoconstrictor activity and inhibition of oxygen uptake in perfused rat hindlimb. *Acta Physiol. Scand.* **161**(2)**:** 161-169.

Rattigan S, Clark M, Barrett E (1997b). Hemodynamic actions of insulin in rat skeletal muscle: evidence for capillary recruitment. *Diabetes* **46**(9)**:** 1381-1388.

Rattigan S, Clark M, Barrett E (1999). Acute vasoconstriction-induced insulin resistance in rat muscle in vivo. *Diabetes* **48**(3)**:** 564-569.

Rattigan S, Dora K, Colquhoun E, Clark M (1993). Serotonin-mediated acute insulin resistance in the perfused rat hindlimb but not in incubated muscle: a role for the vascular system. *Life Sci.* **53**(20)**:** 1545-1555.

Rattigan S, Dora KA, Colquhoun EQ, Clark MG (1995). Inhibition of insulin-mediated glucose uptake in rat hindlimb by an alpha-adrenergic vascular effect. *Am J Physiol Endocrinol Metab* **268**(2)**:** E305-E311.

Rich S, McLaughlin VV (2003). Endothelin receptor blockers in cardiovascular disease. *Circulation* **108**(18)**:** 2184-2190.

Richards S, Dora K, Rattigan S, Colquhoun E, Clark M (1993). Role of extracellular UTP in the release of uracil from vasoconstricted hindlimb. *American Journal of Physiology Heart and Circulation Physiology* **264**(1)**:** H233-H237.

Richter EA, Ruderman NB, Gavras H, Belur ER, Galbo H (1982). Muscle glycogenolysis during exercise: dual control by epinephrine and contractions. *Am J Physiol Endocrinol Metab* **242**(1)**:** E25-32.

Romanko O, Ali M, Mintz J, Stepp D (2009). Insulin resistance impairs endothelial function but not adrenergic reactivity or vascular structure in fructose-fed rats. *Microcirculation* **16**(5)**:** 414-423.

Romanko OP, Stepp DW (2005). Reduced constrictor reactivity balances impaired vasodilation in the mesenteric circulation of the obese Zucker rat. *Am J Physiol Heart Circ Physiol* **289**(5)**:** H2097-2102.

Ross BD (1972). *Perfusion techniques in biochemistry: a laboratory manual.* edn. Clarendon Press: Oxford.

Ross R (2007). Exercise and Insulin: Muscle Haemodynamics and Metabolism. Doctor of Philosophy, University of Tasmania, Hobart.

Ross RM, Kolka CM, Rattigan S, Clark MG (2007). Acute blockade by endothelin-1 of haemodynamic insulin action in rats. *Diabetologia* **50**(2)**:** 443-451.

Ruderman N, Houghton C, Hems R (1971). Evaluation of the isolated perfused rat hindquarter for the study of muscle metabolism. *Biochem. J.* **124**(3)**:** 639-651.

Saito T, Jones CC, Huang S, Czech MP, Pilch PF (2007). The Interaction of Akt with APPL1 Is Required for Insulin-stimulated Glut4 Translocation. *J. Biol. Chem.* **282**(44)**:** 32280-32287.

Santuré M, Pitre M, Marette A, Deshaies Y, Lemieux C, Lariviére R, Nadeau A, Bachelard H (2002). Induction of insulin resistance by high-sucrose feeding does not raise mean arterial blood pressure but impairs haemodynamic responses to insulin in rats. *Br. J. Pharmacol.* **137**(2)**:** 185-196.

Satoh H, Nguyen MTA, Trujillo M, Imamura T, Usui I, Scherer PE, Olefsky JM (2005). Adenovirus-Mediated Adiponectin Expression Augments Skeletal Muscle Insulin Sensitivity in Male Wistar Rats. *Diabetes* **54**(5)**:** 1304-1313.

Schäffler A, Langmann T, Palitzsch KD, Schölmerich J, Schmitz G (1998). Identification and characterization of the human adipocyte apM-1 promoter. *Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression* **1399**(2-3)**:** 187-197.

Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF (1995). A novel serum protein similar to C1q, produced exclusively in adipocytes. *J. Biol. Chem.* **270**(45)**:** 26746-26749.

Schroeder CA, Jr., Chen Y-L, Messina EJ (1999). Inhibition of NO synthesis or endothelium removal reveals a vasoconstrictor effect of insulin on isolated arterioles. *Am J Physiol Heart Circ Physiol* **276**(3)**:** H815-820.

Shapiro L, Scherer PE (1998). The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. *Curr. Biol.* **8**(6)**:** 335-340.

Shemyakin A, Salehzadeh F, Bohm F, Al-Khalili L, Gonon A, Wagner H, Efendic S, Krook A, Pernow J (2010). Regulation of Glucose Uptake by Endothelin-1 in Human Skeletal Muscle in Vivo and in Vitro. *J. Clin. Endocrinol. Metab.* **95**(5)**:** jc.2009-1506.

Shichiri M, Hirata Y, Ando K, Emori T, Ohta K, Kimoto S, Ogura M, Inoue A, Marumo F (1990). Plasma endothelin levels in hypertension and chronic renal failure. *Hypertension* **15**(5)**:** 493-496.

Shklyaev S, Aslanidi G, Tennant M, Prima V, Kohlbrenner E, Kroutov V, Campbell-Thompson M, Crawford J, Shek EW, Scarpace PJ, Zolotukhin S (2003). Sustained peripheral expression of transgene adiponectin offsets the development of diet-induced obesity in rats. *Proc. Natl. Acad. Sci. U. S. A.* **100**(24)**:** 14217-14222.

Simandle SA, Erdös B, Snipes JA, Miller AW, Busija DW (2005). Insulin resistance does not impair contractile responses of cerebral arteries. *Life Sci.* **77**(18)**:** 2262-2272.

Spranger J, Verma S, Gohring I, Bobbert T, Seifert J, Sindler AL, Pfeiffer A, Hileman SM, Tschop M, Banks WA (2006). Adiponectin Does Not Cross the Blood-Brain Barrier but Modifies Cytokine Expression of Brain Endothelial Cells. *Diabetes* **55**(1)**:** 141-147.

St-Pierre P, Genders AJ, Keske MA, Richards SM, Rattigan S (2010). Loss of insulin-mediated microvascular perfusion in skeletal muscle is associated with the development of insulin resistance. *Diabetes, Obesity and Metabolism* **12**(9)**:** 798-805.

Stefan N, Stumvoll M, Vozarova B, Weyer C, Funahashi T, Matsuzawa Y, Bogardus C, Tataranni PA (2003). Plasma Adiponectin and Endogenous Glucose Production in Humans. *Diabetes Care* **26**(12)**:** 3315-3319.

Steinberg H, Baron A (2002). Vascular function, insulin resistance and fatty acids. *Diabetologia* **V45**(5)**:** 623-634.

Steinberg H, Brechtel G, Johnson A, Fineberg N, Baron A (1994). Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J. Clin. Invest.* **94**(3)**:** 1172-1179.

Steinberg HO, Paradisi G, Hook G, Crowder K, Cronin J, Baron AD (2000). Free fatty acid elevation impairs insulin-mediated vasodilation and nitric oxide production. *Diabetes* **49**(7)**:** 1231-1238.

Studier FW, Moffatt BA (1986). Use of Bacteriophage-T7 Rna-Polymerase to Direct Selective High-Level Expression of Cloned Genes. *J. Mol. Biol.* **189**(1)**:** 113-130.

Sugita H, Kaneki M, Tokunaga E, Sugita M, Koike C, Yasuhara S, Tompkins R, Martyn J (2002). Inducible nitric oxide synthase plays a role in LPS-induced hyperglycemia and insulin resistance. *American Journal of Physiology Endocrinology and Metabolism* **282**(2)**:** E386-394.

Sweeney T, Sarelius I (1989). Arteriolar control of capillary cell flow in striated muscle. *Circ. Res.* **64**(1)**:** 112-120.

Symons JD, McMillin SL, Riehle C, Tanner J, Palionyte M, Hillas E, Jones D, Cooksey RC, Birnbaum MJ, McClain DA, Zhang Q-J, Gale D, Wilson LJ, Abel ED (2009). Contribution of Insulin and Akt1 Signaling to Endothelial Nitric Oxide Synthase in the Regulation of Endothelial Function and Blood Pressure. *Circ. Res.* **104**(9)**:** 1085-1094.

Takahashi K, Ghatei MA, Lam H-C, O'Halloran DJ, Bloom SR (1990). Elevated plasma endothelin in patients with diabetes mellitus. *Diabetologia* **33**(5)**:** 306-310.

Takeuchi T, Adachi Y, Ohtsuki Y, Furihata M (2007). Adiponectin receptors, with special focus on the role of the third receptor, T-cadherin, in vascular disease. *Medical Molecular Morphology* **40**(3)**:** 115-120.

Tan GD, Debard C, Funahashi T, Humphreys SM, Matsuzawa Y, Frayn KN, Karpe F, Vidal H (2005). Changes in adiponectin receptor expression in muscle and adipose tissue of type 2 diabetic patients during rosiglitazone therapy. *Diabetologia* **48**(8)**:** 1585-1589.

Tan KCB, Xu A, Chow WS, Lam MCW, Ai VHG, Tam SCF, Lam KSL (2004). Hypoadiponectinemia Is Associated with Impaired Endothelium-Dependent Vasodilation. *J. Clin. Endocrinol. Metab.* **89**(2)**:** 765-769.

Tao L, Gao E, Jiao X, Yuan Y, Li S, Christopher TA, Lopez BL, Koch W, Chan L, Goldstein BJ, Ma XL (2007). Adiponectin Cardioprotection After Myocardial Ischemia/Reperfusion Involves the Reduction of Oxidative/Nitrative Stress. *Circulation* **115**(11)**:** 1408-1416.

Taubert D, Rosenkranz A, Berkels R, Roesen R, Schömig E (2004). Acute effects of glucose and insulin on vascular endothelium. *Diabetologia* **47**(12)**:** 2059-2071.

Thai TL, Churchill GC, Arendshorst WJ (2009). NAADP receptors mediate calcium signaling stimulated by endothelin-1 and norepinephrine in renal afferent arterioles. *Am J Physiol Renal Physiol* **297**(2)**:** F510-516.

Tietge UJF, Boker KHW, Manns MP, Bahr MJ (2004). Elevated circulating adiponectin levels in liver cirrhosis are associated with reduced liver function and altered hepatic hemodynamics. *Am J Physiol Endocrinol Metab* **287**(1)**:** E82-89.

Tomas E, Tsao T-S, Saha AK, Murrey HE, Zhang Cc, Itani SI, Lodish HF, Ruderman NB (2002). Enhanced muscle fat oxidation and glucose transport by Acrp30 globular domain: Acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proc. Natl. Acad. Sci. U. S. A.* **99**(25)**:** 16309-16313.

Tooke J, Goh K (1998). Endotheliopathy precedes type 2 diabetes. *Diabetes Care* **21**(12)**:** 2047-2049.

Trayhurn P, Wang B, Wood IS (2008). Hypoxia in adipose tissue: a basis for the dysregulation of tissue function in obesity? *Br. J. Nutr.* **100**(02)**:** 227-235.

Tsao T-S, Murrey HE, Hug C, Lee DH, Lodish HF (2002). Oligomerization state-dependent activation of NF-kappa B signaling pathway by adipocyte complement-related protein of 30 kDa (Acrp30). *J. Biol. Chem.* **277**(33)**:** 29359-29362.

Tsao T-S, Tomas E, Murrey HE, Hug C, Lee DH, Ruderman NB, Heuser JE, Lodish HF (2003). Role of disulfide bonds in Acrp30/Adiponectin structure and signaling specificity: Different oligomers activate different signal tranduction pathways. *J. Biol. Chem.* **278**(50)**:** 50810-50817.

Tsuchida A, Yamauchi T, Ito Y, Hada Y, Maki T, Takekawa S, Kamon J, Kobayashi M, Suzuki R, Hara K, Kubota N, Terauchi Y, Froguel P, Nakae J, Kasuga M, Accili D, Tobe K, Ueki K, Nagai R, Kadowaki T (2004). Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity. *J. Biol. Chem.* **279**(29)**:** 30817-30822.

van Harmelen V, Eriksson A, Astrom G, Wahlen K, Naslund E, Karpe F, Frayn K, Olsson T, Andersson J, Ryden M, Arner P (2008). Vascular Peptide Endothelin-1 Links Fat Accumulation With Alterations of Visceral Adipocyte Lipolysis. *Diabetes* **57**(2)**:** 378-386.

Vayssettes-Courchay C, Bouysset F, Verbeuren T (2005). Sympathetic activation and tachycardia in lipopolysaccharide treated rats are temporally correlated and unrelated to the baroreflex. *Autonomic Neuroscience* **120**(1-2)**:** 35-45.

Verma S, Yao L, Stewart DJ, Dumont AS, Anderson TJ, McNeill JH (2001). Endothelin antagonism uncovers insulin-mediated vasorelaxation in vitro and in vivo. *Hypertension* **37**(2)**:** 328-333.

Vincent MA, Barrett EJ, Lindner JR, Clark MG, Rattigan S (2003). Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. *Am J Physiol Endocrinol Metab* **285**(1)**:** E123-129.

Vincent MA, Clerk LH, Lindner JR, Klibanov AL, Clark MG, Rattigan S, Barrett EJ (2004). Microvascular recruitment is an early insulin effect that regulates skeletal muscle glucose uptake in vivo. *Diabetes* **53**(6)**:** 1418-1423.

Vincent MA, Dawson D, Clark ADH, Lindner JR, Rattigan S, Clark MG, Barrett EJ (2002). Skeletal muscle microvascular recruitment by physiological hyperinsulinemia precedes increases in total blood flow. *Diabetes* **51**(1)**:** 42-48.

Vincent MA, Rattigan S, Clark MG (2001a). Size-Dependent Effects of Microspheres on Vasoconstrictor-Mediated Change in Oxygen Uptake by Perfused Rat Hindlimb. *Microvasc. Res.* **62**(3)**:** 306-314.

Vincent MA, Rattigan S, Clark MG, Bernard SL, Glenny RW (2001b). Spatial Distribution of Nutritive and Nonnutritive Vascular Routes in Perfused Rat Hindlimb Muscle Using Microspheres. *Microvasc. Res.* **61**(1)**:** 111-121.

Vollus GC, Bradley EA, Roberts MK, Newman JMB, Richards SM, Rattigan S, Barrett EJ, Clark MG (2007). Graded occlusion of perfused rat muscle vasculature decreases insulin action. *Clin. Sci.* **112**(8)**:** 457-466.

Wagner-Mann C, Sturek M (1991). Endothelin mediates Ca influx and release in porcine coronary smooth muscle cells. *American Journal of Physiology - Cell Physiology* **260**(4)**:** C771-777.

Waki H, Yamauchi T, Kamon J, Ito Y, Uchida S, Kita S, Hara K, Hada Y, Vasseur F, Froguel P, Kimura S, Nagai R, Kadowaki T (2003). Impaired multimerization of human adiponectin mutants associated with diabetes: Molecular structure and multimer formation of adiponectin. *J. Biol. Chem.* **278**(41)**:** 40352-40363.

Waki H, Yamauchi T, Kamon J, Kita S, Ito Y, Hada Y, Uchida S, Tsuchida A, Takekawa S, Kadowaki T (2005). Generation of globular fragment of adiponectin by leukocyte elastase secreted by monocytic cell line THP-1. *Endocrinology* **146**(2)**:** 790-796.

Wallis MG, Wheatley CM, Rattigan S, Barrett EJ, Clark ADH, Clark MG (2002). Insulin-mediated hemodynamic changes are impaired in muscle of zucker obese rats. *Diabetes* **51**(12)**:** 3492-3498.

Wang Y, Lam K, Xu J, Lu G, Xu L, Cooper G, Xu A (2005). Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner. *J. Biol. Chem.* **280**(18)**:** 18341-18347.

Wang Y, Lam KSL, Chan L, Chan KW, Lam JBB, Lam MC, Hoo RCL, Mak WWN, Cooper GJS, Xu A (2006). Post-translational modifications of the four conserved lysine residues within the collagenous domain of adiponectin are required for the formation of its high molecular weight oligomeric complex. *J. Biol. Chem.* **281**(24)**:** 16391-16400.

Wang Y, Lu G, Wong WPS, Vliegenthart JFG, Gerwig GJ, Lam KSL, Cooper GJS, Xu A (2004). Proteomic and functional characterization of endogenous adiponectin purified from fetal bovine serum. *Proteomics* **4**(12)**:** 3933-3942.

Wang Y, Xu A, Knight C, Xu LY, Cooper GJS (2002). Hydroxylation and glycosylation of the four conserved lysine residues in the collagenous domain of

adiponectin. Potential role in the modulation of its insulin-sensitizing activity. *J. Biol. Chem.* **277**(22)**:** 19521-19529.

Webb RC (2003). Smooth muscle contraction and relaxation. *Adv Physiol Educ* **27**(4)**:** 201-206.

Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA (2001). Hypoadiponectinemia in obesity and type 2 diabetes: Close association with insulin resistance and hyperinsulinemia. *J. Clin. Endocrinol. Metab.* **86**(5)**:** 1930-1935.

Wheatley CM, Rattigan S, Richards SM, Barrett EJ, Clark MG (2004). Skeletal muscle contraction stimulates capillary recruitment and glucose uptake in insulin-resistant obese Zucker rats. *Am J Physiol Endocrinol Metab* **287**(4)**:** E804-809.

Whitehead JP, Richards AA, Hickman IJ, Macdonald GA, Prins JB (2006). Adiponectin - a key adipokine in the metabolic syndrome. *Diabetes, Obesity and Metabolism* **8**(3)**:** 264-280.

Wild S, Roglic G, Green A, Sicree R, King H (2004). Global Prevalence of Diabetes: Estimates for the year 2000 and projections for 2030. *Diabetes Care* **27**(5)**:** 1047-1053.

Wilkes JJ, Hevener A, Olefsky J (2003). Chronic endothelin-1 treatment leads to insulin resistance in vivo. *Diabetes* **52**(8)**:** 1904-1909.

Womack L, Peters D, Barrett E, Kaul S, Price W, Lindner J (2009). Abnormal skeletal muscle capillary recruitment during exercise in patients with type 2 diabetes mellitus and microvascular complications. *J. Am. Coll. Cardiol.* **53**(23)**:** 2175-2183.

Wu X, Motoshima H, Mahadev K, Stalker TJ, Scalia R, Goldstein BJ (2003). Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. *Diabetes* **52**(6)**:** 1355-1363.

Xi W, Satoh H, Kase H, Suzuki K, Hattori Y (2005). Stimulated HSP90 binding to eNOS and activation of the PI3-Akt pathway contribute to globular adiponectin-induced NO production: Vasorelaxation in response to globular adiponectin. *Biochem. Biophys. Res. Commun.* **332**(1)**:** 200-205.

Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn B, Kadowaki T (2002). Adiponectin stimulates glucose

utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat. Med.* **8**(11)**:** 1288-1295.

Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, Hioki K, Uchida S, Ito Y, Takakuwa K, Matsui J, Takata M, Eto K, Terauchi Y, Komeda K, Tsunoda M, Murakami K, Ohnishi Y, Naitoh T, Yamamura K, Ueyama Y, Froguel P, Kimura S, Nagai R, Kadowaki T (2003). Globular Adiponectin Protected ob/ob Mice from Diabetes and ApoE-deficient Mice from Atherosclerosis. *J. Biol. Chem.* **278**(4)**:** 2461-2468.

Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman M, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T (2001). The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat. Med.* **7**(8)**:** 941-946.

Yanagisawa M, Kurihara H, Kimura S, Goto K, Masaki T (1988). A novel peptide vasoconstrictor, endothelin, is produced by vascular endothelium and modulates smooth muscle Ca2+ channels. *J Hypertens Suppl.* **6**(4)**:** S188-191.

Yang W-S, Lee W-J, Funahashi T, Tanaka S, Matsuzawa Y, Chao C-L, Chen C-L, Tai T-Y, Chuang L-M (2001). Weight Reduction Increases Plasma Levels of an Adipose-Derived Anti-Inflammatory Protein, Adiponectin. *J. Clin. Endocrinol. Metab.* **86**(8)**:** 3815-3819.

Yang Z, Li J (2008). Stimulation of endothelin-1 gene expression by insulin via phosphoinositide-3 kinase-glycogen synthase kinase-3beta signaling in endothelial cells. *Life Sci.* **82**(9-10)**:** 512-518.

Yano W, Kubota N, Itoh S, Kubota T, Awazawa M, Moroi M, Sugi K, Takamoto I, Ogata H, Tokuyama K, Noda T, Terauchi Y, Ueki K, Kadowaki T (2008). Molecular Mechanism of Moderate Insulin Resistance in Adiponectin-Knockout Mice. *Endocr. J.* **55**(3)**:** 515-522.

Yki-Jarvinen H, Utriainen, T. (1998). Insulin-induced vasodilatation: physiology or pharmacology? *Diabetologia* **41**(4)**:** 369-379.

Yoda-Murakami M, Taniguchi M, Takahashi K, Kawamata S, Saito K, Choi-Miura N-H, Tomita M (2001). Change in Expression of GBP28/Adiponectin in Carbon Tetrachloride-Administrated Mouse Liver. *Biochem. Biophys. Res. Commun.* **285**(2)**:** 372-377.

Yoshimoto S, Ishizaki Y, Sasaki T, Murota S (1991). Effect of carbon dioxide and oxygen on endothelin production by cultured porcine cerebral endothelial cells. *Stroke* **22**(3)**:** 378-383.

Youd J, Rattigan S, Clark M (2000). Acute impairment of insulin-mediated capillary recruitment and glucose uptake in rat skeletal muscle in vivo by TNF-alpha. *Diabetes* **49**(11)**:** 1904-1909.

Zhang L, Vincent MA, Richards SM, Clerk LH, Rattigan S, Clark MG, Barrett EJ (2004). Insulin Sensitivity of Muscle Capillary Recruitment In Vivo. *Diabetes* **53**(2)**:** 447-453.

Zhang L, Wheatley CM, Richards SM, Barrett EJ, Clark MG, Rattigan S (2003). TNF-α acutely inhibits vascular effects of physiological but not high insulin or contraction. *Am J Physiol Endocrinol Metab* **285**(3)**:** E654-660.

Zhou L, Deepa SS, Etzler JC, Ryu J, Mao X, Fang Q, Liu DD, Torres JM, Jia W, Lechleiter JD, Liu F, Dong LQ (2009). Adiponectin Activates AMP-activated Protein Kinase in Muscle Cells via APPL1/LKB1-dependent and Phospholipase C/Ca2+/Ca2+/Calmodulin-dependent Protein Kinase Kinase-dependent Pathways. *J. Biol. Chem.* **284**(33)**:** 22426-22435.